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DIETARY ADMINISTRATION OF ETHANOL AND METHANOL EXTRACTS OF *Withania somnifera* ROOT STIMULATES INNATE IMMUNITY, PHYSIOLOGICAL PARAMETERS AND GROWTH IN NILE TILAPIA *Oreochromis niloticus*

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ABSTRACT

Efficacy of ethanol and methanol extracts of medicinal herb *Withania somnifera* roots was evaluated in Nile tilapia *Oreochromis niloticus* on immunostimulation and growth. *Withania* roots were extracted with water, ethanol, methanol, methylene chloride, hexane, successive methanol. Phenol, flavonoid content and antiradical activity of all the extracts were measured. Ethanol extract showed the highest phenol, flavonoid content and antiradical activity followed by methanol extract. Subsequently, Nile tilapia (n = 126) were fed diets containing different concentrations (0.0, 0.3, 0.5 and 0.7 g kg^{-1} feed) of ethanol and methanol extracts of *W. somnifera* roots for 30 days (3 replicates). Fish fed plant extract fortified diets showed significantly better ($P<0.05$) immunological, haematological, biochemical and growth parameters compared to the fish fed control diet. Fish fed diet containing ethanol extract at the concentration of 0.7 g kg^{-1} feed showed the highest immunological (phagocytotic activity, respiratory burst activity, serum lysozyme, total protein, total immunoglobulin), haematological (total red blood cells, haemoglobin, hematocrit, total white blood cells, lymphocyte), biochemical (reduced glutathione, glutathione reductase activity) and growth (final weight, weight gain, daily weight gain, specific growth rate) parameters. The plant extract might act as potent free radical scavenger in fish tissues and have tissue protecting ability, thus increasing fish health.

How to Cite

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INTRODUCTION

Nile tilapia *Oreochromis niloticus* is one of the most important fish species cultured intensively throughout the world (Soliman and Yacout, 2016). Such intensive culture induces stress, suppresses immune system and increases chance of infectious diseases in fish (Harikrishnan et al., 2011). A wide variety of chemotherapeutic agents are used as immunostimulants to promote fish health and control different fish diseases. In teleosts, immunostimulants might enhance phagocytotic cell activity, lymphocyte activation, complement system activation, lysozyme activity and antibody production (Chakraborty and Hancz, 2011). However, large scale application of synthetic antibiotics and chemotherapeutics in fish culture may cause development of antibiotic-resistant bacteria, environmental pollution and accumulation of antibiotic residues in fish (Singer et al., 2016). Considering the regulations on use of chemicals in food fish, dependency on synthetic antibiotics and chemotherapeutics may cause uncertainty on the future of intensive tilapia culture (Doan et al., 2017a; Doan et al., 2017b). Therefore, the use of natural immunostimulants may be considered as suitable alternative for augmented tilapia production.

Application of plant extracts as natural immunostimulants in fish has attained considerable interest during last few years (Chakraborty and Hancz, 2011). Plant extracts contain different bioactive constituents that are reported to enhance growth, feed consumption, act as immunostimulating and antistress tonic, and promote antimicrobial properties in fish (Chakraborty and Hancz, 2011; Chakraborty et al., 2014). These plant bioactive compounds may be a useful source of new medicines that enhance fish production and health, maintain food safety and quality, and conserve aquatic environment. Plants having higher antioxidant activity or radical scavenging potential are a good source to provide immunostimulation or immunomodulation, to help protect against several diseases, and reduce reactive oxygen species formation and its associated damage (Samad et al., 2014; Boudjeko et al., 2015). The antiradical activity of plant extract is often found to be associated with the presence of phenols and flavonoids as principal phytoconstituent (Carvalho et al., 2017). However, the nature of phytoconstituents in plant extracts and their immunostimulating properties are reported to depend on extracting solvent and concentration of the extract (Dhanani et al., 2017).

Withania somnifera (L. Dunal), a fast growing Indian herb, is reported to have anabolizing and virilizing effects (Raju et al., 2017). The plant has been found to possess medicinal values and various therapeutic uses in mammals (Dar et al., 2016). Various studies have indicated the immunomodulatory effects of withanolides which is the principle phytoconstituent of the plant (Chandrasekaran et al., 2017). Dietary administration of *W. somnifera* root powder was reported to stimulate immunity and disease resistance in rohu carp *Labeo rohita* (Sharma et al., 2010). However, limited studies are available concerning application of *W. somnifera* plant extracts as stimulators of innate immunity, general health and growth in fish (Murthy and Kiran, 2013). Considering this aspect, the present study was aimed to comprehensively evaluate the immunostimulating efficacy of different solvent extracts of *W. somnifera* roots during its dietary administration at various concentrations in Nile tilapia. Different non-specific immune, biochemical, haematological and growth parameters were measured to determine the ideal extracting solvent and dietary concentration of the plant extract for its *in vivo* application during Nile tilapia culture.

MATERIALS AND METHODS

Preparation of plant extracts

W. somnifera roots were purchased from the Gariahat market (Kolkata, West Bengal), rinsed in distilled water, shade-dried and powdered. Plant powder (250 g) was individually extracted with 500 mL of different solvents (hexane, methylene chloride, methanol, ethanol, water) in a percolator. Each extract was dried under pressure at 45°C using a rotary vacuum evaporator and stored at -20°C in amber glass bottle (Hussain et al., 2009). For successive methanol extraction, plant powder (200 g) was extracted by maceration under gentle agitation in a glass vessel for 48 h at room temperature, using successively hexane (200 mL for 5 h, 3 times), dichloromethane (200 mL for 5 h, 3 times) and methanol (200 mL for 5 h, 3 times) (Moundipa et al., 2005). Finally, the methanol extract was dried and stored at -20°C.

Qualitative phytochemical studies of plant extracts

Standard procedures were followed to screen presence of various phytochemical groups in different solvent extracts of *Withania* roots (Malpani et al., 2011; Kumar and Bhardwaj, 2012; Ray et al., 2013).

Determination of total phenol content of plant extracts

Standard procedure was performed using Folin-Ciocalteu reagent to determine total phenol content of plant extracts (Maisuthisakul et al., 2007).

Determination of total flavonoid content of plant extracts

A colorimetric assay was used following standard protocol to evaluate total flavonoid content of plant extracts (Maisuthisakul et al., 2007).

Study of antioxidant properties of plant extracts

The antioxidant activity of the plant extracts was evaluated using stable radical DPPH by standard methods (Maisuthisakul et al., 2007).

Dietary treatment of Nile tilapia with different solvent extracts of the plant

Nile tilapia (mean weight 25.00 ± 0.40 g) was transported to the laboratory from the fish hatchery in oxygen packing and kept in 2000-liter concrete tank for acclimatization to laboratory conditions. No fish mortality was recorded during acclimatization. After 10 days of acclimatization, fish was randomly divided into 7 groups of 18 fish each (n=126). Fish in each treatment group was distributed in three 140-liter glass aquaria (6 fish / aquaria) and the aquaria were maintained at a constant water temperature (27-28°C), pH (7.3-7.8), dissolved oxygen (5.0-6.0 mg L^{-1}) and under similar photoperiod (14 L: 10 D). Water in all aquaria was replaced daily. Fish in seven treatment groups were fed diets containing different solvent extracts of *W. somnifera* roots at different concentrations as follows:

Group 1: Control (without plant extract)

Group 2: Ethanol extract of *W. somnifera* roots at concentration of 0.3 g kg^{-1} feed

Group 3: Ethanol extract of *W. somnifera* roots at concentration of 0.5 g kg^{-1} feed

Group 4: Ethanol extract of *W. somnifera* roots at concentration of 0.7 g kg^{-1} feed

Group 5: Methanol extract of *W. somnifera* roots at concentration of 0.3 g kg^{-1} feed

Group 6: Methanol extract of *W. somnifera* roots at concentration of 0.5 g kg^{-1} feed

Group 7: Methanol extract of *W. somnifera* roots at concentration of 0.7 g kg^{-1} feed

Extracts at desired concentrations were dissolved in dimethyl sulfoxide (DMSO) (Moundipa et al., 2005) and added to finely ground (<500-1000 µm) commercially available floating fish feed (Tokyu® Fish Food Spirulina, Tokyu, Japan). The ingredients and feed composition of this commercial fish feed according to the manufacturer as printed on packets are presented in Table 1.

The control feed was prepared by adding only DMSO without any plant extract. The feed was then wetted with deionized water and thorough mixing was done. Finally it was pelleted with a pelleter (diameter 2 mm) and dried at room temperature. Fish were hand-fed experimental diets twice daily at the rate of 5% body weight day^{-1} for 30 days.

Study of cellular immune parameters

Fish from different treatment groups (6 fish / group) were anesthetized with phenoxy-ethanol (1:20000, v / v) and blood samples were collected from the caudal vein using a sterile insulin syringe containing one drop of heparin as an anticoagulant. Leukocytes from each blood sample were isolated by density gradient centrifugation (Chakraborty et al., 2015) and cellular immune parameters, such as respiratory burst activity and phagocytosis assay, were measured using standard protocol. Respiratory burst activity was quantified by the nitroblue tetrazolium (NBT) assay (Secombes 1990), while phagocytosis activity of the leukocytes was measured spectrophotometrically by the method of Seeley et al. (1990).

Study of humoral immune parameters

Total sera lysozyme (µg mL^{-1}), total serum protein (mg mL^{-1}) and total immunoglobulin (mg mL^{-1}) were determined in plasma samples from fish of different experimental groups, following previously described standard methods (Sankaran and Gurnani, 1972; Chakraborty et al., 2015).

Table 1. Ingredients and feed composition of the artificial fish feed (Tokyu® Fish Food Spirulina, Tokyu, Japan) according to the manufacturer as printed on packets

Ingredients	White fish meal, wheat flour, shrimp meal, dried yeast, soybean meal, wheat germ meal, dehydrated alfalfa meal
Vitamins	A, C, D, E, K, B_1, B_2, B_3, B_{12}, Inositol, Nicotinic acid, Ca-Pantothenate Choline, Biotion, Carotenoid, Para-Amino Benzoic Acid, Follic Acid
Minerals	Iron (Fe), Copper (Cu), Zinc (Zn), Manganese (Mn), Cobalt (Co), Phosphorous (P), Magnesium (Mg), P-Amino Benzoic Acid (Paba)
Special Ingredients	Carotenoids, NS Garm, Chlorophyll
Feed composition	
Crude Protein	Min. 32%
Crude Fat	Min. 4%
Crude Ash	Min. 10%
Moisture	Max. 9%
Nitrogen-free Extract	Min. 31%

Study of hematological parameters

Haematological parameters such as total count, differential count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), total serum glucose and albumin were analyzed using standard protocols (Min and Kang, 2008).

Study of biochemical parameters

Serum non-enzymatic and enzymatic antioxidant parameters such as reduced glutathione (GSH), malonaldehyde (MDA), glutathione reductase (GRD), glutathione S-transferase activity (GST) and serum liver enzymes such as alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) were analyzed in fish from different treatment groups (6 fish / group), using standard protocols (Min and Kang, 2008; Padmini et al., 2009; Bamidele et al., 2010, Das et al., 2017).

Study of growth parameters

After 30 days, weight of all fish from different treatment groups was measured individually. Growth parameters such as weight gain (g), daily weight gain (g day^{-1}), specific growth rate (SGR, % day^{-1}) and feed conversion ratio (FCR) were calculated for different treatment groups using standard formula (Pechsiri and Yakupitiyage, 2005) as follows:

Weight gain (g) = Mean final weight (g) – Mean initial weight (g)

Daily weight gain (g day^{-1}) = [Mean final weight (g) – Mean initial weight (g)] / days

SGR (SGR, % day^{-1}) = [(ln final weight – ln initial weight) / time (days)] ×100

FCR = Total amount of dry feed consumed (g) / wet weight gain of fish (g)

Statistical analyses

All data were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was performed to analyze different parameters, followed by a Tukey test in case significant differences were found between means. IBM SPSS Statistics Version 20 software was used to conduct all statistical analysis.

RESULTS

Qualitative phytochemical analysis

W. somnifera roots extracted with all solvents showed presence of alkaloids and steroid / terpenoids, while no extracts contained glycoside. All solvent extracts except aqueous contained flavonoids, while tannin and saponin were present in all solvent extracts except methylene chloride and hexane. Carbohydrate was present only in methanolic extract (Table 2).

Table 2. Qualitative analysis of phytochemicals in different solvent extracts of *W. somnifera* roots

Solvent for extraction	Phytochemical groups						
	Tannin	Saponin	Alkaloid	Carbohydrate	Glycoside	Flavonoid	*Steroid / Terpenoid*
Aqueous	+	+	+	-	-	-	+
Ethanol	+	+	+	-	-	+	+
Methanol	+	+	+	+	-	+	+
Methylene chloride	-	-	+	-	-	+	+
Hexane	-	-	+	-	-	+	+
Successive methanol	+	+	+	-	-	+	+

Total phenol, flavonoid and antioxidant properties

Ethanol extract of *W. somnifera* roots showed the highest antiradical activity, phenol content and flavonoid content, which were significantly higher (*P<0.05*) compared to those in all other solvent extracts (Table 3).

The second highest antiradical activity, phenol content and flavonoid content were observed for methanol extract of the plant material. The lowest antiradical activity (0.01 ± 0.00%) was observed in methylene chloride extract, while aqueous extract of the plant material showed the lowest phenol content (14.17 ± 0.61 mg of GAE g^{-1} dry weight) and flavonoid content (0.08 ± 0.005 mg of RE g^{-1} dry weight) (Table 3). There was a weak correlation (R^2 = 0.57) between the phenol content and flavonoid content, but a strong correlation was observed between antiradical activity and phenol content (R^2 = 0.77), and between antiradical activity and flavonoid content (R^2 = 0.79) (Fig. 1).

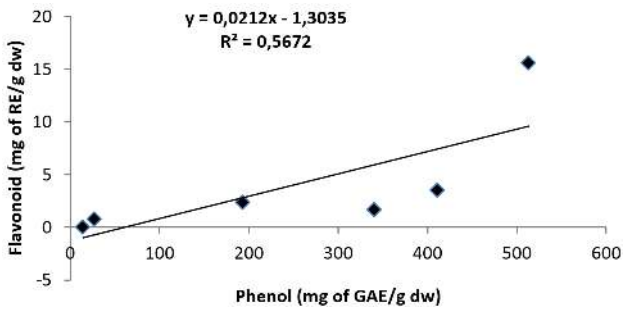
Humoral and cellular immune parameters

Most humoral and cellular immune parameters increased significantly (*P<0.05*) in fish fed *W. somnifera* root extracts fortified diets compared to those in fish fed control diet (Table 4). A dose-dependent increase in phagocytotic activity and respiratory burst activity was observed for both ethanol and methanol extracts treatment groups. Fish fed diets containing ethanol extract of *W. somnifera* roots at a particular concentration showed significantly higher (*P<0.05*) immunological parameters than the corresponding concentration group for methanol extract of the plant material (Table 4). Treatment Group 4 showed the highest phagocytotic activity, respiratory burst activity, sera lysozyme content, total serum protein and total immunoglobulin among all the treatment groups (Table 4).

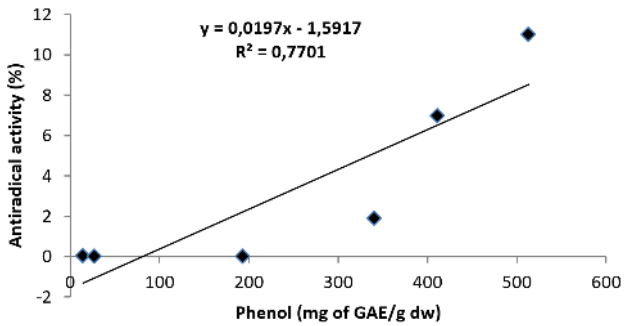
Table 3. Antiradical activity, phenol content and flavonoid content of different solvent extracts from *W. somnifera* roots

Plant	Solvent for Extraction	Antiradical activity (%)	Phenol Content (mg of GAE g^{-1} dry weight)	Flavonoid Content (mg of RE g^{-1} dry weight)
W. somnifera roots	Aqueous	0.05 ± 0.00[a]	14.17 ± 0.61[a]	0.08 ± 0.01[a]
	Methanol	6.99 ± 0.06[c]	410.96 ± 0.98[e]	3.49 ± 0.02[e]
	Ethanol	10.99 ± 0.41[d]	513.01 ± 1.66[f]	15.56 ± 0.31[f]
	Hexane	0.04 ± 0.00[a]	26.97 ± 0.55[b]	0.75 ± 0.02[b]
	Methylene chloride	0.01 ± 0.00[a]	193.00 ± 4.04[c]	2.39 ± 0.01[d]
	Successive	1.90 ± 0.01[b]	339.87 ± 1.10[d]	1.67 ± 0.04[c]

All data are expressed in mean ± SEM. Different superscripts mark significant difference (*P<0.05*) in means within column.



a)



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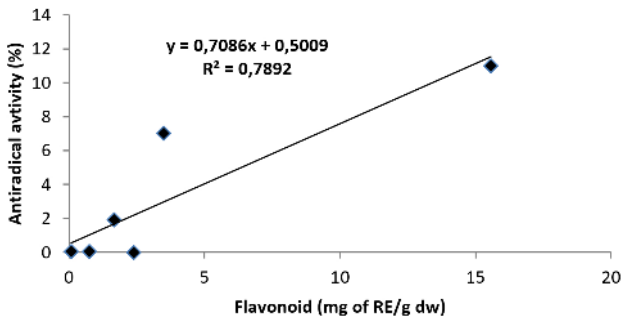

c)

Fig 1. Correlation between total phenolic content and total flavonoid content (a), total phenolic content and antiradical activity (b) and total flavonoid content and antiradical activity (c) of different solvent extracts of *W. somnifera* roots

Hematological parameters

The results of the haematological parameters at the end of 30-day dietary treatment with plant material are given in Table 5. RBC indices such as total RBC count, hemoglobin concentration and hematocrit increased significantly (*P<0.05*) in fish fed diet fortified with ethanol *Withania* 0.7 g kg^{-1} feed (Group 4) compared to those in fish fed control diet (Group 1). Group 4 showed a significant decrease (*P<0.05*) in MCV compared to Group 1, while no significant difference (*P>0.05*) was observed between Group 1 and Group 4 for MCHC and MCH (Table 5). Total WBC count, lymphocyte, monocyte and eosinophil count increased significantly (*P<0.05*) in Group 4 compared to those in Group 1. There was no significant difference (*P>0.05*) in neutrophil count, serum glucose and serum albumin between Group 1 and Group 4 (Table 5). Fish fed control diet showed the lowest WBC count.

Biochemical parameters

The non-enzymatic antioxidant parameter MDA level decreased, while GSH level increased significantly (*P<0.05*) in fish fed plant extract containing diets compared to those in fish fed control diet. Enzymatic antioxidant GRD level has increased, but GST level decreased significantly (*P<0.05*) in plant extracts treatment groups compared to those in control fish (Table 6). Treatment group 4 showed the lowest MDA and GST levels, and the highest GSH and GRD levels. A dose-dependent alteration pattern was observed for the biochemical parameters within the ethanol and methanol extract treatment groups (Table 6). Serum level of liver enzymes ALP, GOT and GPT decreased significantly (*P<0.05*) in fish fed *W. somnifera* root extract diet compared to those in fish fed control diet (Table 6).

Growth performance

Fish fed diets containing ethanol and methanol extracts of *W. somnifera* roots at different concentrations showed significantly higher final weight than control (*P<0.05*). The highest final weight was achieved in fish fed diet fortified with ethanol *Withania* 0.7 g kg^{-1} feed. This treatment

Table 4. Comparative immunological parameters of tilapia fed control, different concentrations of ethanol and different concentrations of methanol extracts of *W. somnifera* roots supplemented diets after 30 days of culture

Immunological Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Phagocytotic Activity (OD_{510})	0.39 ± 0.001^{a}	0.69 ± 0.002^{e}	0.75 ± 0.001^{f}	0.82 ± 0.002^{g}	0.48 ± 0.001^{b}	0.64 ± 0.002^{c}	0.67 ± 0.002^{d}
Respiratory Burst Activity(OD_{610})	0.07 ± 0.001^{a}	0.14 ± 0.003^{cd}	0.15 ± 0.003^{d}	0.16 ± 0.003^{e}	0.10 ± 0.003^{b}	0.12 ± 0.002^{c}	0.14 ± 0.001^{d}
Sera Lysozyme (µg mL^{-1})	19.67 ± 1.14^{a}	33.09 ± 0.23^{c}	33.69 ± 0.52^{c}	35.72 ± 0.53^{c}	20.91 ± 0.22^{a}	21.99 ± 0.24^{a}	26.98 ± 1.96^{b}
Total Serum Protein (mg mL^{-1})	0.12 ± 0.001^{a}	0.21 ± 0.010^{cd}	0.21 ± 0.002^{cd}	0.23 ± 0.004^{e}	0.19 ± 0.001^{b}	0.20 ± 0.005^{c}	0.21 ± 0.002^{de}
Total Immunoglobulin (mg mL^{-1})	0.07 ± 0.002^{a}	0.10 ± 0.010^{cd}	0.10 ± 0.004^{cd}	0.11 ± 0.003^{d}	0.07 ± 0.004^{ab}	0.07 ± 0.002^{ab}	0.09 ± 0.002^{bc}

All data are expressed in mean ± SEM. Different superscripts mark significant difference (*P<0.05*) in means within rows.

Table 5. Comparative haematological parameters of tilapia fed control, different concentrations of ethanol and different concentrations of methanol extracts of *W. somnifera* roots supplemented diets after 30 days of culture

Haematological parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Total RBC (106 mL^{-1})	1.84 ± 0.01^{ab}	1.97 ± 0.03^{bc}	2.09 ± 0.03^{cd}	2.18 ± 0.04^{d}	1.76 ± 0.06^{a}	1.82 ± 0.04^{ab}	1.90 ± 0.02^{ab}
Hemoglobin (g dL^{-1})	8.41 ± 0.08^{a}	8.95 ± 0.09^{bc}	9.06 ± 0.10^{c}	9.08 ± 0.06^{c}	8.57 ± 0.06^{a}	8.61 ± 0.04^{ab}	8.55 ± 0.08^{a}
Hematocrit (%)	25.09 ± 0.03^{a}	26.43 ± 0.32^{bc}	26.43 ± 0.17^{bc}	26.80 ± 0.09^{c}	25.43 ± 0.27^{a}	25.66 ± 0.06^{ab}	25.43 ± 0.17^{a}
MCHC (g dL^{-1})	33.50 ± 0.32^{a}	33.86 ± 0.12^{a}	34.27 ± 0.59^{a}	33.55 ± 0.26^{a}	33.72 ± 0.60^{a}	33.70 ± 0.07^{a}	33.63 ± 0.52^{a}
MCH (pg)	45.62 ± 0.75^{abc}	48.95 ± 2.08^{c}	47.38 ± 1.35^{bc}	45.03 ± 0.57^{abc}	41.61 ± 0.41^{a}	43.43 ± 1.03^{ab}	45.50 ± 0.46^{abc}
MCV (µm^{3})	136.15 ± 1.03^{bc}	134.40 ± 1.81^{abc}	126.70 ± 1.30^{ab}	122.83 ± 1.58^{a}	145.01 ± 3.64^{c}	140.60 ± 3.68^{c}	133.91 ± 2.27^{abc}
Total WBC (103 mL^{-1})	20.19 ± 0.20^{a}	20.78 ± 0.02^{ab}	21.57 ± 0.15^{ab}	23.78 ± 0.19^{c}	20.75 ± 0.35^{ab}	21.60 ± 0.44^{ab}	21.93 ± 0.60^{b}
Lymphocyte (%)	92.62 ± 0.34^{a}	93.70 ± 0.53^{abc}	94.47 ± 0.61^{bc}	94.82 ± 0.50^{c}	92.96 ± 0.00^{ab}	93.04 ± 0.05^{abc}	93.00 ± 0.09^{abc}
Monocyte (%)	6.00 ± 0.15^{a}	7.02 ± 0.08^{b}	7.20 ± 0.57^{b}	7.36 ± 0.08^{b}	5.56 ± 0.39^{a}	5.99 ± 0.14^{a}	5.89 ± 0.21^{a}
Neutrophil (%)	4.55 ± 0.04^{a}	4.74 ± 0.07^{a}	4.87 ± 0.07^{a}	5.09 ± 0.14^{a}	4.52 ± 0.01^{a}	4.70 ± 0.25^{a}	5.18 ± 0.32^{a}
Eosinophil (%)	0.54 ± 0.03^{a}	0.86 ± 0.08^{c}	0.74 ± 0.01^{bc}	0.73 ± 0.02^{bc}	0.52 ± 0.03^{a}	0.58 ± 0.01^{ab}	0.65 ± 0.03^{ab}
Serum Glucose (mg mL^{-1})	5.35 ± 0.20^{b}	5.21 ± 0.04^{ab}	5.27 ± 0.11^{ab}	5.39 ± 0.14^{b}	4.60 ± 0.21^{a}	4.98 ± 0.14^{ab}	5.16 ± 0.04^{ab}
Serum Albumin (mg mL^{-1})	1.14 ± 0.07^{ab}	0.94 ± 0.02^{a}	1.10 ± 0.06^{ab}	1.22 ± 0.07^{b}	0.96 ± 0.01^{a}	0.99 ± 0.02^{a}	1.24 ± 0.04^{b}

All data are expressed in mean ± SEM. Different superscripts mark significant difference (*P<0.05*) in means within rows.

Table 6. Comparative serum biochemical parameters of tilapia fed control, different concentrations of ethanol and different concentrations of methanol extracts of *W. somnifera* roots supplemented diets after 30 days of culture

Serum biochemical parameters (U mg protein^{-1})	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
MDA	14.49 ± 0.30^{d}	10.66 ± 0.29^{b}	10.42 ± 0.14^{ab}	9.40 ± 0.17^{a}	12.54 ± 0.38^{c}	12.30 ± 0.15^{c}	12.13 ± 0.29^{c}
GSH	20.62 ± 0.12^{a}	23.41 ± 0.53^{ab}	24.80 ± 1.85^{bc}	27.67 ± 0.38^{c}	23.51 ± 0.58^{ab}	23.63 ± 0.47^{ab}	25.73 ± 0.33^{bc}
GRD	253.71 ± 0.81^{a}	265.18 ± 0.62^{b}	268.87 ± 2.35^{bc}	270.90 ± 0.73^{c}	267.54 ± 0.85^{bc}	268.20 ± 0.52^{bc}	270.28 ± 0.53^{bc}
GST	1.85 ± 0.06^{c}	1.69 ± 0.03^{b}	1.64 ± 0.02^{ab}	1.53 ± 0.02^{a}	1.68 ± 0.02^{b}	1.65 ± 0.02^{ab}	1.58 ± 0.03^{ab}
ALP	0.31 ± 0.01^{c}	0.26 ± 0.01^{bc}	0.25 ± 0.01^{b}	0.19 ± 0.01^{a}	0.26 ± 0.01^{bc}	0.23 ± 0.01^{ab}	0.24 ± 0.01^{ab}
GOT	22.60 ± 0.47^{e}	8.66 ± 0.31^{a}	12.55 ± 0.57^{b}	14.75 ± 1.23^{b}	19.60 ± 0.91^{de}	18.24 ± 0.55^{cd}	15.32 ± 0.58^{bc}
GPT	188.06 ± 1.25^{e}	176.96 ± 1.16^{cd}	172.03 ± 0.76^{ab}	169.91 ± 0.82^{a}	174.39 ± 0.48^{bc}	180.76 ± 0.56^{d}	174.39 ± 0.91^{bc}

All data are expressed in mean ± SEM. Different superscripts mark significant difference (*P<0.05*) in means within rows.

Table 7. Comparative growth parameters of tilapia fed control, different concentrations of ethanol and different concentrations of methanol extracts of *W. somnifera* roots supplemented diets after 30 days of culture

Growth parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Final Weight (g)	50.34 ± 0.98^{a}	60.14 ± 0.37^{bcd}	63.31 ± 0.62^{de}	66.85 ± 1.40^{e}	56.83 ± 0.38^{b}	58.69 ± 0.52^{bc}	62.36 ± 0.91^{cde}
Weight Gain (g)	25.34 ± 0.59^{a}	35.14 ± 0.03^{cd}	38.31 ± 0.23^{d}	41.85 ± 1.00^{e}	31.83 ± 0.78^{b}	33.69 ± 0.12^{bc}	37.37 ± 0.52^{d}
Daily Weight Gain (g day^{-1})	0.84 ± 0.02^{a}	1.17 ± 0.00^{cd}	1.28 ± 0.01^{d}	1.40 ± 0.03^{e}	1.06 ± 0.03^{b}	1.12 ± 0.00^{bc}	1.25 ± 0.02^{d}
SGR (% day^{-1})	2.33 ± 0.06^{a}	2.93 ± 0.02^{cd}	3.10 ± 0.00^{e}	3.28 ± 0.05^{f}	2.74 ± 0.09^{b}	2.84 ± 0.01^{bc}	3.05 ± 0.02^{de}
FCR	1.48 ± 2.04^{b}	1.07 ± 0.01^{a}	0.98 ± 0.03^{a}	0.90 ± 0.10^{a}	1.18 ± 0.31^{a}	1.11 ± 0.03^{a}	1.00 ± 0.08^{a}

All data are expressed in mean ± SEM. Initial weight of the fish were 25.00 ± 0.40 g. Different superscripts mark significant difference (P<0.05) in means within rows.

group (Group 4) showed a significantly higher (*P<0.05*) weight gain (g), daily weight gain (g day^{-1}) and SGR (% day^{-1}) compared to all other treatment groups as well (Table 7). FCR values between the groups fed different concentrations of plant extract fortified diets (Group 2 – Group 7) showed statistical homogeneity. Treatment with ethanol extract at 0.7 g kg^{-1} feed (Group 4), which showed the best immunostimulating and growth properties, also showed the lowest FCR (Table 7).

DISCUSSION

The main objective of the present study was to improve the non-specific immune system and general health of Nile tilapia using a natural plant product as immunostimulant and health promoting agent. Plants having higher antioxidant activity are a good source to provide immunostimulation, help fight against several diseases and reduce reactive oxygen species formation and its associated damage (Chakraborty and Hancz 2011; Hoseinifar et al., 2017; Hoseinifar et al., 2018). Dietary administration of myrtle powder and medlar leaf extract was reported to improve mucosal immune parameters, growth performances, and to alter mRNA levels of growth, antioxidant and immune related genes in zebra fish and common carp, respectively (Safari et al., 2017; Hoseinifar et al., 2017). Withanolides, a group of phytosteroid present in *W. somnifera* roots as principal phytoconstituent, have been reported to possess immunostimulatory activity (Chandrasekaran et al., 2017). Compounds such as flavonoids and alkaloids associated with antioxidant activity, saponin and glycosides associated with growth, hematological modulation and stimulation of gonadotrophin secretion, triterpenoids associated with promoting muscle growth and phytosterol associated with elevating plasma vitellogenin level were observed to be present in different solvent extracts of *W. somnifera* roots (Chakraborty et al., 2014). Quantitative estimation of flavonoids and phenols, and antiradical activity of different solvent extracts of *W. somnifera* roots revealed that ethanol and methanol extracts might have the highest antioxidant property (Table 3). The regression analysis showed a strong correlation between the antiradical activity with both phenol and flavonoid content in the plant. Similar correlation between the phenol and flavonoid content in plant extract with antiradical activity was also observed for other medicinal plants (Mukherjee et al., 2017; Kähkönen et al., 2001). Considering these results, both the ethanol and methanol extracts were selected for subsequent in vivo study. Dietary administration of both ethanol and methanol extracts of *W. somnifera* root was found to stimulate innate immunity and growth in Nile tilapia. However, ethanol extract of the plant material was observed to be more effective.

The phagocytotic activity of leukocytes is a primary defense mechanism and an important characteristic of the nonspecific innate immune system in fish (Jeney et al., 2009). Many herbs that contain different phytochemicals, mainly flavonoids, are observed to enhance intracellular respiratory burst activity in leukocytes of fishes (Chakraborty and Hancz, 2011). The increase of phagocytotic activity, respiratory burst and lysozyme activity, as observed in the present study, might be due to the increase in neutrophils and monocytes in the blood of fish fed plant extract fortified diet compared to those in fish fed control diet (Pratheepa and Sukumaran, 2014). *Oreochromis mossambicus* fed diets containing acetone extract of *W. somnifera* showed improvement in growth, immune activity and survival in an earlier study (Immanuel et al., 2009). Jeney et al. (2009) have reported variable results regarding the effect of immunostimulating herbs on total protein and immunoglobulin levels in fish. In the present study, dietary administration of *W. somnifera* root extracts significantly increased (*P<0.05*) total protein and total immunoglobulin level of plasma. Plant constituents are suggested to directly activate innate defense mechanisms by acting on receptors, triggering gene activation, consequently resulting in production of anti-microbial molecules (Bricknell and Dalmo, 2005). Thus it might be postulated that at least part of the stimulatory capacities of *W. somnifera* ethanol and methanol extracts might be associated with one or more components present in it. Flavonoids from dietary sources have often been associated with various health benefits (Panche et al., 2016) and the highest immunostimulating activities of ethanol extract of *W. somnifera* roots might be associated with the highest flavonoid content and antiradical activity of the extract. In the present study, the immunostimulating efficacy of the ethanol extract of the plant was observed to be dose-dependent. Sharma et al., 2010 reported that dietary administration of *W. somnifera* root powder at different concentrations resulted in stimulation of immunological parameters and increase in disease resistance, but not in a dose-dependent manner. However, several other studies have indicated dose-dependent functions of many plant extracts in animals (Shabbir et al., 2016; Lundstrom et al., 2017). *Withania* root powder was reported to have stimulatory effect on immunological parameters and increase disease resistance in *Labeo rohita* against pathogen (Pandey et al., 2012). Enhancement of the RBC indices and WBC indices in fish fed diets fortified with ethanol extract of *Withania somnifera* indicated that the extract might have erythropoietic and lymphopoietic attributes, and might be used as adaptogen in Nile tilapia culture (Pratheepa and Sukumaran, 2014). Serum albumin level has often been regarded as unspecific indicator of nutritional status (Cabrerizo et al., 2015). No significant difference in serum albumen level between fish fed control and plant extract fortified diets indicated that the extracts might have no anti-nutritional property.

Extent of lipid peroxidation is known to be a sensitive

indicator of damage to various tissues under different environmental conditions (Verlecar et al., 2007). In this study, comparatively lower MDA levels in fish fed plant extract fortified diets might be indicative of the better general health of those fish than the control ones. Being one of the major regulators of the intracellular redox state, GSH plays an important role in the non-enzymatic defense system (Préville et al., 1999). Increase in GSH level is associated with growth stimulation by nutrients and growth factors (Diaz-Vivancos et al., 2015). Fish belonging to the treatment Group 4 that showed the highest GSH level in this study, showed the highest growth as well, indicating the role of the plant extract in stimulating fish health. Enzymes such as GRD and GST play an important role in cellular antioxidant defense and important processes of metabolic pathway, thereby retain the reduced form of glutathione within threshold level and maintain GSH:GSSG ratio (Mukherjee et al., 2017). Increase in GRD coupled with decrease in GST levels in fish fed plant extract supplemented diets might be indicative of the antioxidative effects of the plant extracts. Altogether, the plant extract might act as potent free radical scavenger in fish tissues, which in turn reduced the production of reactive oxygen species (ROS) in cells and stimulated fish health and growth.

Enzymes such as ALP, GOT and GPT are confined to the cells of liver, heart, gill, kidney, muscles and other organs. The enzymes are important factors in assessing liver cytolysis and their existence in the serum might provide information on organ dysfunction (Ozer et al., 2008). Fish in a polluted aquatic system was reported to show high level of serum ALP, GOT and GPT, indicating tissue damage (Abalaka, 2013). In this study the levels of all these enzymes were found to be significantly lower ($P<0.05$) in fish fed plant extract supplemented diets compared to the control group (Table 6). Such results indicate that the plant extract might have tissue protecting ability and thus might increase general fish health.

The antioxidant activity and maintenance of redox balance by the phytochemicals present in the ethanol extracts may stimulate growth in fish. Phytoconstituents are reported to stimulate immunity, act as substrate for biochemical reaction, inhibitors of enzymatic reactions, enhance the absorption and provide stability of essential nutrients in the intestine. These are found to promote DNA, RNA, protein synthesis and to stimulate GH and IGF1 production. Thus, phytochemicals might influence the growth of fish during dietary supplementation (Chakraborty and Hancz, 2014). The significant growth increase in fish fed diets supplemented with *W. somnifera* root extracts might be attributed to the general good health of the fish.

The results suggest that *W. somnifera* possess immunostimulating and growth promoting efficacy. Considering the immunological, haematological, biochemical and growth parameters, it may be postulated that the ideal concentration for dietary administration of *W. somnifera* is ethanol extract at the concentration of 0.7 g kg^{-1} feed. The extract may have a potential role as dietary supplement in fish feed augmenting fish health and thus in the development of sustainable eco-friendly aquaculture. However, further investigation regarding the mechanism of immunostimulation and growth induction by the plant extract in fish is warranted.

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SAŽETAK

EKSTRAKTI METANOLA I ETANOLA KORJENA BILJKE *Withania somnifera* U RIBLJOJ HRANI STIMULIRAJU IMUNITET, FIZIOLOŠKE POKAZATELJE I RAST NILSKE TILAPIJE *Oreochromis niloticus*

U istraživanju je procijenjena učinkovitost etanola i metanolnih ekstrakata korijena ljekovite biljke *Withania somnifera* na imunostimulativna svojstva i rast Nilske tilapije *Oreochromis niloticus*. Korijeni Withanie ekstrahirani su vodom, etanolom, metanolom, metilen-kloridom, heksanom i sukcesivnim metanolom. Izmjereni su sadržaji fenola i flavonoida te antiradikalna aktivnost svih ekstrakata. Nakon toga, izvršena je hranidba nilske tilapije (n=126) hranom koja je sadržavala različite koncentracije (0, 0,3, 0,5 i 0,7 g kg^{-1} hrane) etanola i metanolnih ekstrakata korijena *W. somnifera* tijekom 30 dana kroz 3 ponavljanja. Pri usporedbi s kontrolnom hranidbom, ribe hranjene hranom obogaćenom biljnim ekstraktom indicirale su značajno bolje ($P <0,05$) imunološke, hematološke i biokemijske pokazatelje te parametre rasta. Riba hranjena hranom koja sadrži ekstrakt etanola u koncentraciji od 0,7 g kg^{-1} imala je najveće imunološke (fagocitna aktivnost, aktivnost respiratornog praska, lizozim u serumu, ukupni protein, ukupni imunoglobulin), hematološke (ukupna crvena krvna zrnca, hemoglobin, hematokrit, ukupno bijelih krvnih zrnaca, limfocita) i biokemijske (smanjena aktivnost glutationa, glutation-reduktaza) pokazatelje te najviše parametre rasta (masa, prirast, specifičnu stopu rasta). Prema rezultatima, istraživani biljni ekstrakt može djelovati kao sredstvo za uklanjanje slobodnih radikala u ribljim tkivima te posjeduje sposobnost zaštite tkiva, pritom povećavajući zdravlje riba.

Ključne riječi: Ekstrakt etanola, ekstrakt metanola, imunostimulacija, rast, antioksidans

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