Evaluation of phenolic extract of licorice roots in diets of Nile tilapia (*Oreochromis niloticus*)

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Aims
The aims of this study were to evaluate the effect of methanolic extract of licorice roots (MELRs) as a feed additive on the growth performance, feed utilization, and innate immunity of Nile tilapia (*Oreochromis niloticus)*, and to screen the phenolic compounds contained in the different MELRs through reverse-phase high-performance liquid chromatography (HPLC).

Background
Licorice is doubtless one of the most popular medicinal plants, with its roots being the most used part. Particularly, the species *Glycyrrhiza glabra* L. has well-known therapeutic properties, which have been documented since the Egyptian age.

Materials and methods
MELR was prepared as follows: in a typical experiment, 500 g of the plant was dried at room temperature. The dried plant was defatted separately with *n*-hexane in a Soxhlet apparatus for 15 h. The plant residue was then extracted with methanol in a Soxhlet apparatus for 20 h. The obtained data were analyzed using one-way procedure SPSS according to the following model: $Y_{ij} = \mu + T_i + e_{ij}$, where μ is the over mean, T_i is the fixed effect of the rocket supplementation (1…4), and e_{ij} is random error. The differences between the experimental groups were determined using Duncan's multiple range test.

Results and discussion
Screening the phenolic compounds contained in the different MELR achieved through reverse-phase HPLC analysis showed 21 phenolic and flavonoid compounds. The MELR supplementation enhanced fish growth compared with the control diet, and the highest fish performance was obtained at 0.04% MELR level. Supplementation with MELR was found to have an antibacterial activity antagonistic to pathogenic bacteria *Aeromonas jandaei* infection in fish. It is recommended that 0.04% MELR be used as a feed additive for Nile tilapia to enhance its growth performance, health, and innate immunity.

Conclusion
This study was carried out to evaluate the effect of MELRs as a feed additive on the growth performance, feed utilization, and innate immunity of Nile tilapia. MELR enhances fish growth rate, feed utilization, and innate immunity. The highest fish growth was obtained when fish were fed a diet containing 0.04% extract.

Keywords:
feed utilization, growth performance, high-performance liquid chromatography, innate immunity, methanolic extract of licorice roots, Nile tilapia

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Introduction

Licorice is doubtless one of the most popular medicinal plants, with its roots being the most used part. Particularly, the species *Glycyrrhiza glabra* L. has well-known therapeutic properties, which have been documented since the Egyptian age. The extracts from licorice leaves investigated in our study appear to be good candidates as multifactorial chemopreventive agents in humans [1]. From a chemical point of view, licorice roots contain several triterpenes, such as glycyrrhizin and glycyrrhetic acid, together with flavones, isoflavones, chalcones, and several related compounds, which often present as glycosides [2,3]. These compounds are thought to be responsible for the several therapeutic properties ascribed to this plant, whose extracts are used as tonic, detoxicant, expectorant, and as anti-inflammatory, antimicrobial, antiatherogenic, antiallergic, antiviral agents against metabolic syndrome, obesity, and immune system alterations, as well as a source of cosmeceutical ingredient [4].

Nile tilapia (*Oreochromis niloticus*) is a well-known tropical fish native to Africa. It is principally herbivorous, although occasionally omnivorous, and it is an efficient converter of waste foodstuff and appears to thrive well on artificial supplemental

feed [5]. Nile tilapia farming is socially more acceptable and it is technically and economically more viable and sustainable. The attributes that make Nile tilapia highly suitable for fish farming are its general hardiness and tolerance to a wide range of environmental conditions, ease of breeding, rapid growth rate, resistance to stress and disease, ability to efficiently convert a wide range of natural and artificial feed, as well as organic and domestic wastes, into high-quality protein, ability to reproduce easily in captivity, and good taste [6].

Materials and methods

Extraction and sample preparations

In our study, we used methanolic extract of licorice root (MELR). In a typical experiment, 500 g of the plant was dried at room temperature. The dried plant was defatted separately with *n*-hexane in a Soxhlet apparatus for 15 h. The plant residue was then extracted with methanol in a Soxhlet apparatus for 20 h. The extract was then obtained after the removal of the extraction solvents at reduced pressure under vacuum in a rotary apparatus at 40°C. The extract of licorice root weighed 120 g in *n*-hexane. The weight of the extract after evaporation was 35 g for licorice root. The above extracts were stored at +4°C and unsealed immediately before use.

High-performance liquid chromatographic

For the isolation of phenolic and flavonoid compounds, the defatted residues of the licorice roots were extracted individually with methyl alcohols to give methanolic extract. The extract was evaporated under vacuum at room temperature to dryness. The dry sample was dissolved in methanol and subjected to chromatographic analysis with high-performance liquid chromatography (HPLC) with the following specifications: Agilent 1100 series (Agilent Technologies, Waldbronn, Germany), quaternary pump (G1311A), Degasser (G1322A), Thermostated Autosamples (G1329A), variable wave length detector (G1314A); and column: Zorbax 300SB C_{18} column (Agilent Technologies, USA), under gradient conditions, with a flow rate of 0.8 ml/min. The gradient starting with 95% H_2O containing 0.05% formic acid v/v and 5% methanol was kept constant for 10 min, and then the methanolic solvent concentration was changed according to the following order:15, 30, 40, 45, 60, 80%, and then decreased by 5% after 15, 20, 30, 50, 52, 60, and 65 min, respectively. The injection volume was 50 µl and chromatogram was acquired at 280 nm using Agilent 1100 HPLC. Phenolic compounds of plant extract were identified by comparing their retention times with those of pure standards. The results were expressed as percentage of each compound from the total phenolic compounds.

Diet preparation and feeding regime

Four experimental isonitrogenous (30% crude protein) and 7% lipid diets were formulated to contain 0.0 (control), 0.04, 0.08, and 0.12% MELR. Dietary formulation and proximate composition of the experimental diets are shown in Table 1. The dry ingredients of each diet were thoroughly mixed, and 100 ml of water was added per kg diet. Thereafter, the mixture (ingredients and water) was blended using a kitchen blender to make a paste of each diet. Pelleting of each diet was carried out by passing the blended mixture through a laboratory pellet machine with 1-mm diameter die. The pellets were dried in a drying oven for 24 h at 65°C and stored in plastic bags in a refrigerator at −2°C until its use.

Table 1 Ingredients and proximate chemical analysis of the experimental diets (on dry matter basis) containing different levels of methanolic extract of licorice root

Items	MELR (%)			
	Control	0.04	0.08	0.12
Fish meal	12.4	12.4	12.4	12.4
Soybean meal	42	42	42	42
Ground corn	19	19	19	19
Wheat bran	14	14	14	14
Cod fish oil	2.9	2.9	2.9	2.9
Corn oil	2.3	2.3	2.3	2.3
Vitamin premix[a]	1.5	1.5	1.5	1.5
Mineral premix[b]	1.5	1.5	1.5	1.5
Starch	4.4	4.36	4.32	4.28
MELR	0.0	0.04	0.08	0.12
Total	100	100	100	100
Proximate chemical composition				
Dry matter	90.92	90.39	90.53	91.36
Crude protein	29.87	29.75	29.96	29.91
Crude fat	8.18	7.59	7.60	7.63
Ash	9.46	8.55	8.02	7.92
Crude fiber	5.07	5.10	5.43	5.37
NFE[c]	47.42	49.01	48.99	49.17
GE (kcal/100 g)[d]	440.96	441.2	442.44	443.18
P/E ratio	67.73	67.42	67.72	67.49

GE, gross energy; MELR, methanolic extract of licorice root; NFE, nitrogen-free extract; [a]Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2 g; inositol, 100 g; biotin, 0.3 g; pantothenic acid, 100 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200 g; nicotinic acid, 10 g; cyanocobalamin, 0.005 g; a-tocopherol acetate, 20.1 g; menadione, 2 g; retinol palmitate, 100 000 IU; cholecalciferol, 500 000 IU; [b]Mineral premix (g/kg of premix): $CaHPO_4 \cdot 2H_2O$, 727.2; $MgCO_4\ 7H_2O$, 127.5; KCl, 50.0; NaCl, 60.0; $FeC_6H_5O_7 \cdot 3H_2O$, 25.0; $ZnCO_3$, 5.5; $MnCl_2 \cdot 4H_2O$, 2.5; $Cu(OAc)2 \cdot H_2O$, 0.785; $CoCl_3 \cdot 6H_2O$, 0.477; $CaIO_3 \cdot 6H_2O$, 0.295; $CrCl_3 \cdot 6H_2O$, 0.128; $AlCl_3 \cdot 6\ H_2O$, 0.54; Na_2SeO_3, 0.03; [c]NFE = 100–(protein%+crude fat% + ash% + fiber%); [d]GE was calculated from the guidelines of NRC (1993) as 5.65, 9.45, and 4.1 kcal/g for protein, lipids, and carbohydrates, respectively.

Proximate chemical analysis

Proximate chemical analysis of diets was carried out according to the standard methods [7] for dry matter, crude protein, crude fiber, and ash. Nitrogen-free extract was calculated by difference. Gross energy content of the experimental diets was calculated using factors of 5.65, 9.45, and 4.22 kcal/g of crude protein, crude fat, and carbohydrates, respectively [8].

Fish culture technique

Nile tilapia, *O. niloticus* L., were obtained from fish hatchery, Central Laboratory for Aquaculture Research (Abo-Hammad, Egypt). Fish were kept for 2 weeks in an indoor tank for acclimation, during which they were fed a formulated diet containing 30% crude protein. Fish were frozen at −20°C for proximate analysis initially. Acclimated Nile tilapia fingerlings (5.05 ± 0.004 g) were distributed randomly into 12 140-l aquaria comprising 15 fingerlings per aquarium in triplicates. Each aquarium was supplied with compressed air through air-stones using aquarium air pumps. Fishs were fed the tested diets at a feeding rate of 5% of live body weight for the first 4 weeks and 3% of live body weight for the remaining 12 weeks. The diets were offered to each aquarium twice daily for 12 weeks.

Bacterial challenge

At the end of the experimental period, fish of each treatment were divided into two subgroups: the first group was injected intraperitoneally with pathogenic *Aeromonas jandaei* (10^4 cells/ml), which was obtained from the Fish Disease Department, Central Laboratory for Aquaculture Research (Egypt); and the second group was injected intraperitoneal with 0.2 ml of saline solution and used as a control. Both subgroups were kept under observation for 10 days after challenge, during which incidences of daily mortality were recorded. The challenge test was carried out according to the method of Brook and colleagues [9,10].

Statistical analysis

The obtained data were analyzed using one-way procedure (SPSS Inc., Chicago, Illinois, USA) according to the following model: $Y_{ij} = \mu + T_i + e_{ij}$, where μ is the over mean, T_i is the fixed effect of the rocket supplementation (1…4), and e_{ij} is random error. The differences between the experimental groups were determined using Duncan's multiple range test [11].

Results and discussion

Phenolic compound identification from licorice roots using high-performance liquid chromatography

Further screenings of the phenolic compounds contained in the methanolic extract were carried out through reverse-phase HPLC analysis of licorice roots. Phenolic contents of MELRs are summarized in Table 2 and Fig. 1.

Selected phenolics in licorice roots, separated and identified using reversed-phase HPLC, are presented in Fig. 2 (chemical structures).

Growth performance

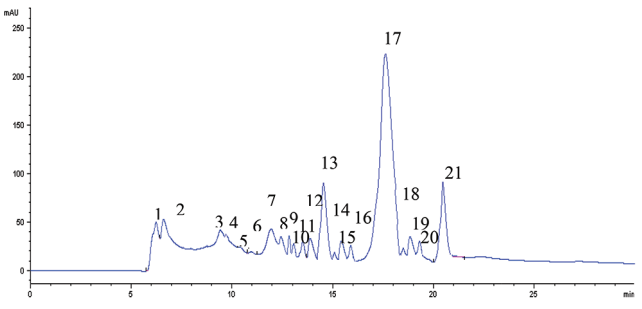
The present study showed that fish fed on diet containing 0.04% MELR showed the highest final weight, average daily gain, relative gain%, and specific growth rate in comparison with the experimental groups (Table 3).

Table 2 Major phenolic compounds (% of total) identified in licorice roots methanolic extract by high-performance liquid chromatography

Number of peaks	Phenolic compounds	Rt (min)	Area%
1	Pyrogallol	6.2	3.12
2	Gallic acid	6.4	3.012
3	Caffeic acid	9.5	4.42
4	Vanillic acid	9.7	2.15
5	Caffeine	10.3	2.021
6	Ferulic acid	11.2	2.014
7	Rutin	11.9	4.011
8	Quercitrin	12.5	2.522
9	P-coumaric acid	12.8	2.12
10	Naringin	13	2.06
11	Benzoic acid	13.5	5.05
12	Ellagic acid	13.9	5.07
13	Liquiritin	14.5	8.12
14	Naringenin	15.2	3.51
15	Myricetin	15.4	5.04
16	Quercetin	15.8	5.14
17	Glycyrrhizinic acid	17.3	20.51
18	Liquiritigenin	18.3	3.41
19	Cinnamic acid	18.8	4.42
20	Apigenin	19.3	4.21
21	Kaempferol	20.5	8.08

Rt, Retention time.

Figure 1



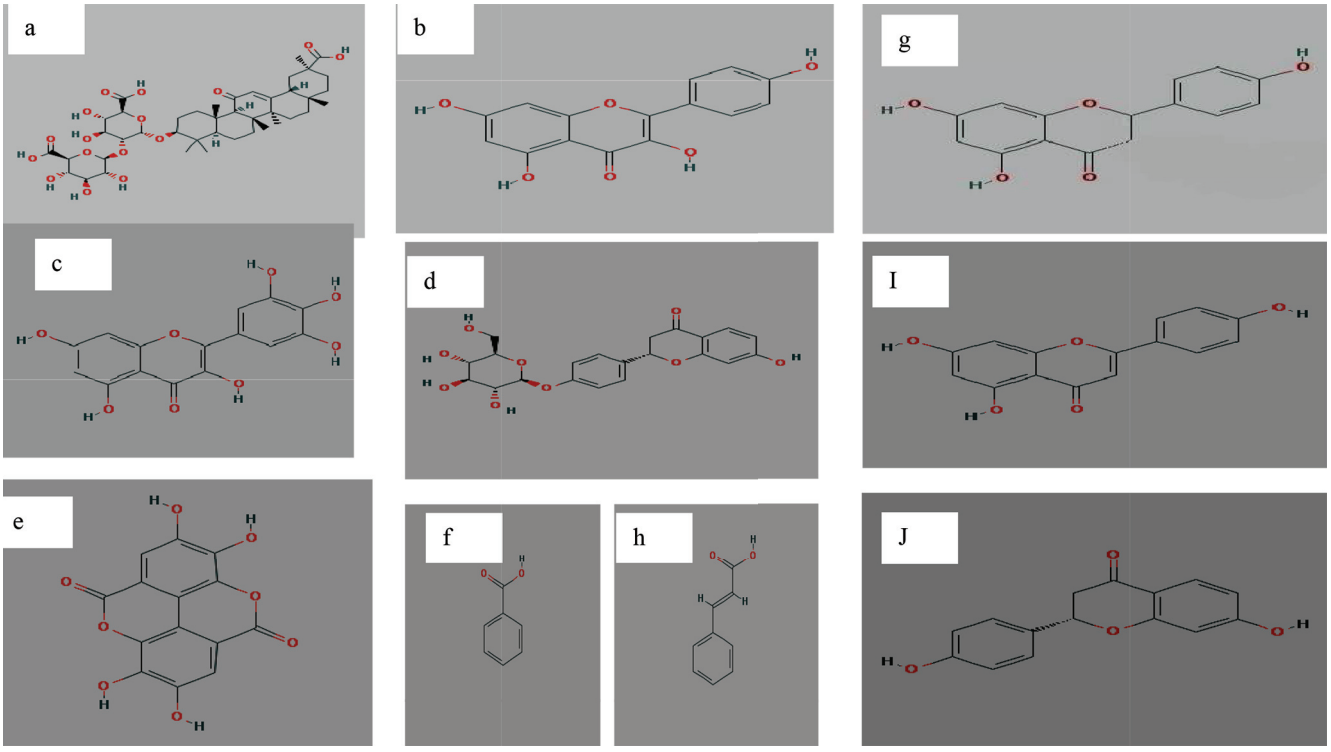
High-performance liquid chromatography chromatogram of licorice root phenolic profile showing the separation of a major chemical component.

The lowest final weight, weight gain, weight gain%, and specific growth rate were observed in fish fed on the control diet. The improved fish growth of Nile tilapia (*O. niloticus*) may be due to its digestive and stimulant effect through their aromatic substances or essential oils that are extracted from their roots and leaves.

Moreover, the improvement in body weight gain is related to the active materials found in plants, causing greater efficiency in the utilization of feed, resulting in enhanced growth. Improvement in the growth performance has been observed in *Litopenaeus vannamei* [12], *Siniperca chuatsi* [13], and *Apostichopus japonicus* (Selenka) [14] fed with glycyrrhizin. These results also agree with that of El-Aidy [15], who showed that supplementing growing Nile tilapia diet with licorice roots significantly improved growth performance compared with the control group.

These results are in agreement with those of Jiang *et al.* [16], who reported that diets supplemented with glycyrrhizinic acid decreased weight gain slightly in the 0.6 g/kg group ($P > 0.05$).

Figure 2



Chemical structure for the most abundant phenolic compounds identified in licorice roots using high-performance liquid chromatography: (a) glycyrrhizinic acid; (b) kaempferol; (c) myricetin; (d) liquiritin; (e) ellagic acid; (f) benzoic acid; (g) naringenin; (h) cinnamic acid; (i) apigenin; (j) liquiritigenin.

Table 3 Growth performance, feed utilization, and survival of Nile tilapia fingerlings fed on diets containing different levels of methanolic extract of licorice root for 12 weeks

Items	MELR (%)DRLM%			
	Control (0.0)	0.04	0.08	0.12
Initial weight (g/fish)	5.25 ± 0.01	5.24 ± 0.01	5.24 ± 0.004	5.26 ± 0.01
Final weight (g/fish)	22.07 ± 0.19^{D}	27.46 ± 0.18^{a}	25.30 ± 0.18^{b}	24.14 ± 0.14^{c}
Average total gain (g/fish)^{e}	16.82 ± 0.19^{D}	22.22 ± 0.17^{a}	20.06 ± 0.18^{b}	18.88 ± 0.13^{c}
Average daily gain (g/fish)^{f}	0.19 ± 0.02	0.25 ± 0.08	0.22 ± 0.10	0.21 ± 0.09
Specific growth rate (%/day)^{g}	1.71 ± 0.01^{D}	1.97 ± 0.01^{a}	1.87 ± 0.01^{b}	1.81 ± 0.004^{c}
Feed intake	29.57 ± 0.28^{C}	32.87 ± 0.20^{a}	31.52 ± 0.22^{b}	30.99 ± 0.18^{b}
Feed conversion ratio^{h}	1.76 ± 0.002^{A}	1.48 ± 0.003^{d}	1.57 ± 0.005^{c}	1.64 ± 0.002^{b}
Protein efficiency ratio^{i}	23.13 ± 0.45	24.42 ± 0.26^{b}	26.87 ± 0.27^{a}	24.52 ± 0.41^{b}

MELR, methanolic extract of licorice root; ^{a,b,c,d}Means with different superscripts in the same row differ significantly $P < 0.05$; ^{e}Average total gain (g/fish) = average final weight (g)–average initial weight (g); ^{f}Average daily gain (g/fish/day) = average total gain (g)/experimental period (days); ^{g}Specific growth rate (%/day) = 100 (ln final weight–ln initial weight/experimental period (days); ^{h}Feed conversion ratio feed intake (g)/live weight gain (g); ^{i}Protein efficiency ratio = live weight gain (g)/protein intake (g).

Feed utilization

Feed intake increased significantly, whereas feed conversion ratio (FCR) improved with supplemented MELR in fish diets (Table 3). Moreover, protein efficiency ratio, apparent protein utilization (APU), and energy utilization (EU) values increased significantly with increasing MELR levels in diets. Increased feed intake resulted from the high demand for nutrients with stimulated growth, or due to improved appetite because of sensory stimulation resulting from the presence of MELR in the diets. The best FCR and higher values of feed intake (FI), protein efficiency ratio, APU, and EU were obtained when fish were fed on diet containing 0.04% MELR. These results are in agreement with that reported by El-Aidy [15], who found that the FCR of all groups fed on licorice root-supplemented diets showed significantly ($P < 0.05$) better FCR (lower) compared with the control group. The improvement was more pronounced with 1% licorice root supplementation level. Ahmed *et al.* [17] showed that feed intake increased significantly, whereas FCR decreased significantly ($P < 0.05$) when fish were fed on cotton seed meal (CSM)-supplemented diets compared with those fed on a control diet. Moreover, they found that the highest and the lowest FCR were obtained at 0.0 (control) and 10 g CSM/kg diet.

Immunity test

After bacterial challenge, fish mortality was 90% in fish fed on control diet, and it was 20–30% in fish fed on MELR diets (Table 4). The experimentally infected fish died with some clinical signs such as tail and fin rot and absence of scale with external skin hemorrhage, and the postmortem finding was septicemic lesions of the internal organs. *A. jandaei* was reisolated from the liver, kidneys, and spleen of the moribund and recently dead fish. nitroblue tetrazolium (NBT) assay was used to determine the activity of phagocytes, especially neutrophils and monocytes. NBT activity increased significantly when fish were fed on 0.04, 0.08, and 0.12% MELR compared with the control group, which had 0% MELR (Table 4). Lysozymes are a family of enzymes with antibacterial activity characterized by the ability to damage the cell wall of bacteria. Lysozyme levels increased with 0.04, 0.08, and 0.12% MELR compared with the fish group fed on control diet (Table 4). Hence, a significant increase in lysozyme activity in the plasma of fish fed for 21 days with diets enriched with 0.04, 0.08, and 0.12% MELR may indicate an increase in the fish defense system against bacterial infection. The results obtained in this study show that MELR increased disease resistance and improved fish survival against experimental infection with *A. jandaei*. Dietary supplementation with licorice extracts to *Ctenopharyngodon idellus* can enhance the resistance to diseases and stress of the fish [14,18]. Both intraperitoneal injection and oral administration of licorice extracts to *Carassius auratus* could enhance the resistance of the fish to stress and *Aeromonas hydrophila* infection [19,20].

Table 4 Change in fish mortality rate after bacterial challenge, respiratory brust, and lysozyme activities of Nile tilapia with different methanolic extract of licorice root levels

Fish groups	Challenge test Mortality (%)	Respiratory burst (NBT) activity	Lysozyme (μg/ml serum)
Control (0.0)	90	0.214	0.186
0.04%	30	0.394	2.122
0.08%	20	0.378	1.767
0.12%	20	0.335	1.667

Conclusion

This study was carried out to evaluate the effect of MELRs as a feed additive on the growth performance, feed utilization, and innate immunity of Nile tilapia. MELR enhances fish growth rate, feed utilization, and innate immunity. The highest fish growth performance was obtained when fish were fed on a diet containing 0.04% extract.

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Conflicts of interest

There are no conflicts of interest.

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