Effects of Roselle calyx (*Hibiscus sabdariffa* L.)-supplemented diets on growth and disease (*Aeromonas hydrophila*) resistance in Nile tilapia (*Oreochromis niloticus* L.)

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Aims
The aim of this study was to determine the effect of phenolic compounds of Roselle calyx (*Hibiscus sabdariffa* Linn) on growth performance, feed utilization, whole-body composition, blood profile, and immunity against *Aeromonas hydrophila* in Nile tilapia, *Oreochromis niloticus*.

Background
H. sabdariffa extracts have demonstrated to have a broad range of therapeutic effects such as antioxidant, antiobesity, anticancer, inhibition of the contractility of rat bladder and uterus, antibacterial, antihypertensive, and antimicrobial activities due to the presence of phenolic compounds.

Materials and methods
Fifteen fish with the same initial weight (5.45 ± 0.01 g/fish) were selected and randomly distributed into four experimental treatments in triplicates. The chemical profiles of phenolic constituents were analyzed using high-performance liquid chromatography to determine the major phenolic compounds.

Results and discussion
The diet containing 1% Roselle calyx showed the highest growth performance and feed utilization in comparison with other dietary treatments. The main phenolic compounds were ferulic acid, rosmarinic acid, apigenin, carnosic acid, cinnamic acid, caffeic acid, chlorogenic acid, p-Coumaric acid, and quercetin-7-*O*-glucoside, in addition to anthocyanins such as delphinidin-3-glucoside, sambubioside, cyanidin-3-monoglucoside, and cyanidin-3-sambubioside.

Conclusion
Roselle calyx was found to have an antibacterial activity antagonistic to pathogenic *A. hydrophila* infection in fish. Lysozyme and bactericidal activities of fish increased significantly in fish fed on Roselle calyx-enriched diets.

Keywords:
growth performance, *Hibiscus sabdariffa* L., high-performance liquid chromatography, phenolic compounds, Roselle calyx

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Introduction

From ancient times, herbs and spices have been used due to their culinary qualities and medicinal properties, including antioxidant activity [1,2]. More recently, the interest in herbs and spices has increased not only for their seasoning and flavoring properties but also for their antioxidant potential. In addition, such property has also demonstrated its importance in the prevention of some diseases. *Aeromonas hydrophila* is one of the most common bacterium that infects koi carp. *A. hydrophila* causes disease known as 'motile aeromonas septicemia,' 'hemorrhagic septicemia,' 'ulcer disease,' or 'red-sore disease' in fish. *A. hydrophila* has been categorized as an opportunistic pathogen. However, the term 'opportunistic pathogen' conveys that *A. hydrophila* is always capable of producing disease if given the chance.

Consumption of herbs and spices has been implicated in the prevention of cardiovascular diseases, carcinogenesis, inflammation, atherosclerosis, etc. [3]. Such properties have been attributed to the presence of several compounds such as vitamins, terpenoids, polyphenols, and flavonoids [4].

Hibiscus is one of the most common flower plants grown worldwide. There are more than 300 species of hibiscus around the world. One of them is *Hibiscus sabdariffa* Linn, which is a member of the Malvaceae family. The origin of *H. sabdariffa* is not fully known,

but it is believed to be a native of tropical Africa. It is known by different synonyms and vernacular names such as Roselle [5,6]. *H. sabdariffa* is an herbaceous plant also known as karkade, Roselle, graines d'oseille, and guinean sorrel [7]. Roselle can be found in almost all warm countries such as India, Saudi Arabia, Malaysia, Thailand, Philippine, Vietnam, Sudan, Egypt, Mali, and Mexico [8,9].

H. sabdariffa extracts have demonstrated to have a broad range of therapeutic effects [10] such as hepatoprotective [11], antioxidant [12,13], antiobesity [14], anticancer [15], inhibition of the contractility of rat bladder and uterus [16], antibacterial [17], antihypertensive [18], and antimicrobial activities due to its phenolic compounds. Different works have shown that *H. sabdariffa* calyx reduces blood pressure in humans, decreasing the viscosity of the blood and stimulating intestinal peristalsis [19,20].

Fish are rich in animal protein of high biological value, polyunsaturated (essential) fatty acids, vitamins, and minerals [21,22]. Its protein content about 15.5% of the world animal protein consumption (30, 20, and 8% for Asia, Africa, and Europe, respectively), as mentioned by National Research Council [22]. Thus, it is the main animal protein source for poor people. Fish are important not only as human food but also as animal feed (fish meal, oil, and silage and fish protein hydrolysates). In addition to its importance as food, they are used in recreational fisheries, as biological controllers, and in scientific research and industries [19].

Materials and methods

The leaves of Roselle calyx were collected from a Zoological Garden, Cairo, Egypt, in May 2012. The plant was identified by Teresa Labib, General Manager and Head of Plant. A voucher specimen (TA_1) has been deposited at the herbarium of the National Research Council.

Phenolic extract of Hibiscus sabdariffa L.

For the isolation of phenolic and flavonoid compounds, defatting was carried out by means of extraction with *n*-hexane in a soxhlet apparatus for 20 h. The two extracts were then obtained after the removal of the extraction solvent at reduced pressure under vacuum in a rotary apparatus at 40°C. The residues of *H. sabdariffa* L. were extracted individually with methyl alcohols to obtain methanolic extract. The extract was evaporated under vacuum at room temperature to dryness.

Two-dimension paper chromatographic investigation

Preliminary phytochemical screening and the two-dimensional paper chromatography of the methanolic extract using two common solvent systems, 15% AcOH (AcOH: H_2O; 15: 85) and BAW (n-BuOH: AcOH: H O; 4: 1: 5 upper layer), revealed the presence of a promising variety of phenolic compounds.

Determination of phenolic compounds using high-performance liquid chromatography

Solutions of available pure standard compounds were dissolved in methanol before injecting in the analytical high-performance liquid chromatography (HPLC) system. Samples were filtered through a 0.45 µm membrane. Analysis of the phenolic compounds in the filtrate was performed using HPLC, Shimadzu Class-VPV 5.03 (Tosoh Bioscience LLC, Kyoto, Japan) equipped with UV-10 A Shimadzu detector, LC-16ADVP binary pump, DCou-14 A degasser, and C_{18} column (Sc 1011 No. H706081). 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. The dry sample was dissolved in methanol and chromatographed under gradient conditions, at 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, and 80%, and then decreased by 5% after 15, 20, 30, 50, 52, 60, and 65 min. The injection volume was 50 µl, and chromatogram was acquired at 280 nm.

Experimental design and dietary treatments

Four experimental diets (30% crude protein and 7% lipid) were formulated containing 0.0 (control), 0.5, 1.0, and 1.5% Roselle calyx of *H. sabdariffa*. The proximate chemical composition of *H. sabdariffa* calyx and the main ingredients of the tested diets are shown in Table 1.

Dietary formulation and proximate composition of the experimental diets are shown in Table 2. The dry ingredients of each diet were thoroughly mixed, and 100 ml of water was added per kg diet. Afterwards, 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 a 1-mm-diameter diet. The pellets were dried in a drying oven for 24 h at 85 °C and stored in plastic bags in a deep freezer at −2 °C until use. The caloric value as digestible energy (DE) of each ingredient was

Table 1 Ingredients and chemical analysis of the experimental diets (on dry matter basis) containing different levels of Roselle calyx

	Control	Roselle calyx levels (%)		
		0.5	1	1.5
Fish meal (HFM)	11	11	11	11
Soybean meal	42.5	42.5	42.5	42.5
Ground cornmeal (CNM)	19.3	19.3	19.3	19.3
Wheat bran	14.9	14.9	14.9	14.9
Cod fish oil	3	3	3	3
Corn oil	2.3	2.3	2.3	2.3
Vitamins premix	1	1	1	1
Minerals premix	2	2	2	2
Starch	4	3.5	3	2.5
Roselle calyx	0.0	0.5	1.0	1.5
Sage plant	0.0	0.0	0.0	0.0
Total	100	100	100	100
Total chemical analysis				
Dry matter	92.23	92.3	92.65	92.42
Crude protein	30.19	30. 85	30.3	30.7
Crude fat	7.18	7.16	7.13	7.11
Ash	7.18	7.27	7.23	7.21
Fiber	5.1	5.3	5.6	5.4
NFE	50.35	49.42	49.74	49.58
GE (kcal/100 g)	444.97	444.69	442.62	444.02
P/E ratio	67.84	69.37	68.45	69.14

Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamine, 0.005 g; α-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100 000 IU; cholecalciferol, 500 000 IU.
Mineral premix (g/kg of premix): $CaHPO_4.2H_2O$, 727.2; $MgCO_4.7H_2O$, 127.5; KCl 50.0; NaCl, 60.0; $FeC_6H_5O_7.3H_2O$, 25.0; $ZnCO_3$, 5.5; $MnCl_2.4H_2O$, 2.5; $Cu(OAc)_2.H_2O$, 0.785; $CoCl_3.6H_2O$, 0.477; $CaIO_3.6H_2O$, 0.295; $CrCl_3.6H_2O$, 0.128; $AlCl_3.6H_2O$, 0.54; Na_2SeO_3, 0.03.
Nitrogen-free extract (calculated by difference)=100–(protein+lipid +ash+fiber).
GE was calculated from National Research Council [22] as 5.65, 9.45, and 4.1 kcal/g for protein, lipid, and carbohydrates, respectively.
GE, gross energy; HFM, herring fish meal; NFE, nitrogen-free extract; P/E ratio, protein to energy ratio.

Table 2 Major phenolic compounds (% of total) identified in Roselle calyx (*Hibiscus sabdariffa*) methanolic extract by high-performance liquid chromatography

Compounds	Retention time	% of major components
Catechin	2.64	7.8
p-Hydroxy benzoic acid	4.35	2.0
Gallic acid	5.72	2.7
Chlorogenic acid	17.12	1.32
Caffeic acid	18.34	7.07
Quinic acid	19.84	1.12
Quercetin	20.10	4.12
p-Coumaric acid	22.32	2.30
Ferulic acid	27.32	11.20
Benzoic acid	30.54	7.30
Cinnamic acid	38.12	5.0
Kaempferol	38.36	18
Ellagic acid	39.72	16
Synergenic acid	40.89	18
Dephinidin-3-glucoside	52	23
Dephinidin-3-sambubioside	53	22
Cyanidin-3-glucoside	54	21
Cyanidin-3-sambubioside	55	21

estimated to be 5.65 kcal DE/g of protein, 9.45 kcal DE/g of lipid, and 4.11 kcal DE/g of carbohydrate [22].

Experimental fish and culture condition

The present study was carried out throughout the Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abou-Hammad, Sharkia, Egypt. The feeding experiment was carried out at the Nutrition Department of CLAR during the year 2014.

All-male Nile tilapia, *Oreochromis niloticus*, fingerlings treated with 17 α-methyl testosterone hormone – the most simple and reliable way to produce all-male tilapia stocks, which consistently grow to a large/more uniform size compared with mixed-sex tilapia – were obtained from the nursery ponds, CLAR, Abbassa, Abu-Hammad, Sharkia, Egypt. The fish were held in a fiberglass tank for 2 weeks for acclimation, during which they were fed a formulated diet containing 30% crude protein. Fifty fish were frozen at −20 °C for initial proximate whole-body analysis. Subsequently, the fish (5.47 ± 0.01 g) were distributed randomly at a rate of 15 fish/140 l aquarium. Each aquarium was aerated using small air pumps. Settled fish wastes along with a half of the aquarium water was siphoned daily, and replaced with well-aerated and dechlorinated tap water from a storage tank. Fish in all treatments were fed the tested diets at a rate of 4% of live body weight for the first 3 weeks, and at a rate of 3% for the rest of the experimental period. Diets were offered twice daily at 9: 00 and 13: 00 h for 12 weeks. Fish in each aquarium were sampled biweekly and the amount of feed adjusted accordingly. Dead fish were daily recorded and removed. At the end of the study, fish were individually weighed.

Growth performance and feed utilization

Fish growth parameters and feed utilization were calculated as follows:

$$\text{Weight gain (g)} = W_2 - W_1.$$

$$\text{Specific growth rate (\%g/day)} = 100\,(\text{Ln}\,W_2 - \text{Ln}\,W_1)/T,$$

where W_1 and W_2 are the initial and final weights, respectively, and T is the experimental period (days).

$$\text{Feed conversion ratio} = \frac{\text{Feed intake}}{\text{Weight gain}}.$$

$$\text{Protein efficiency ratio} = \frac{\text{Weight gain}}{\text{Protein intake}}.$$

$$\text{Apparent protein utilization (\%)} = 100\left[\frac{\text{Protein gain in fish (g)}}{\text{Protein intake in diet (g)}}\right].$$

$$\text{Energy utilization (\%)} = 100\left[\frac{\text{Energy gain in fish (g)}}{\text{Energy intake in diet (g)}}\right].$$

Proximate chemical analysis

Diets and fish were analyzed according to standard methods [22] for moisture, crude protein, total lipids, and ash. Moisture content was estimated by drying samples in an oven at 85°C until constant weight was achieved. Nitrogen content was measured with a micro-Kjeldahl apparatus, and crude protein was estimated by multiplying total nitrogen content by 6.25. Total lipid content was determined by means of ether extraction for 16 h, and ash was determined by combusting samples in a muffle furnace at 550 °C for 6 h. Crude fiber was estimated according to the method of Goering and van Soest [23]. Gross energy was calculated according to the method of National Research Council [22].

Water analysis

Water samples were collected biweekly for chemical analysis. Dissolved oxygen and temperature were measured on site using an oxygen meter (YSI, model 58; Yellow Spring Instrument Co., Yellow Spring, Ohio, USA). Unionized ammonia was measured using a HACH kit (HACH Co., Loveland, Colorado, USA). The pH was measured using a pH meter (Fisher Scientific, Denver, Colorado, USA).

Physiological analysis

At the end of the feeding trial, three fish from each aquarium were taken for physiological investigation. The fish were anesthetized using buffered tricaine methanesulfonate (20 mg/l), and blood was collected from the caudal vein with a sterile syringe and divided equally among three clean and dry tubes. The first part was centrifuged at 3000 *g* for 15 min and the serum was stored at −20 °C for further assays. The second part was mixed with sodium fluoride as an anticoagulant and centrifuged at 3000 *g* for 15 min for separation of plasma for glucose analysis. The last part was mixed with EDTA solution for measuring hemoglobin (Hb), red blood cells (RBCs), and hematocrite (Ht). Hb level was determined colorimetrically using a spectrophotometer according to the method of Stopkopf [24]. Ht was determined using the microhematocrit method [25]. RBCs were determined according to the method described by Natt and Herrick [26]. Total lipid content was determined colorimetrically according to the method of Joseph *et al.*[27]. Total protein content was determined colorimetrically according to the method of Henry [28]. Urea was determined following the method of Patton and Crouch [29]. Creatinine was determined calorimetrically as described by Henry [28]. Glucose was determined colorimetrically following the method of Trinder [30]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined calorimetrically according to the method of Reitman and Frankel [31].

Challenges tests

After the feeding trial, fish of each treatment were divided into two groups. The first one was injected intraperitoneally with pathogenic *A. hydrophila* (0.2×10^{10} cell/ml), which was obtained from the Fish Disease Department, CLAR. The second group was injected intraperitoneally with 0.2 ml of saline solution as a control. Both groups were kept under observation for 10 days and incidences of daily mortality were recorded.

Lysozyme activity

After infection with *A. hydrophila*, lysozyme was estimated based on the turbidity measurements as described by Schaperclaus *et al.*[32]. However, 10 µl of serum was added in the cuvettes to 200 µl of micrococcus suspension (35 mg of micrococcus dry powder/95 ml of 1/15 mol/l phosphate buffer+5.0 ml of NaCl solution). The change in the extinction was measured at 546 nm, by measuring the extinction immediately after adding the solution that contained the lysozyme (start of reaction), and after a 20 min the preparation under investigation was incubated at 40 °C (end of reaction). The lysozyme content was determined based on the calibration curve and the extinction measured.

Serum bactericidal activity

The serum bactericidal activity (SBT) integrated both pharmacokinetic and pharmacodynamic properties in a single set of determinations that examine the ability of

the fish serum. Bacterial cultures of *A. hydrophila* were centrifuged and the pellet was washed and suspended in phosphate buffer saline (PBS). The optical density of the suspension was adjusted to 0.5 at 546 nm. This suspension was serially diluted (1: 10) with PBS five times. SBT was determined by incubating 2 µl of this diluted bacterial suspension with 20 µl of serum in a microvial for 1 h at 37 °C. In the bacterial control group, PBS replaced the serum. After incubation, the number of viable bacteria was determined by counting the colonies after culturing on tryptone soya agar plates for 24 h at 37 °C [32].

Statistical analyses

The obtained data were subjected to one-way analysis of variance. Differences between mean were tested at the 5% probability level using Duncan's new multiple range test. All statistical analyses were carried out using the SPSS program, version 10 (SPSS, Richmond, Virginia, USA), as described by Dytham [33].

Results and discussion

Identification of the phenolic compounds from *Hibiscus sabdariffa* calyx chromatographic investigation

Two-dimension paper chromatographic investigation

Two-dimensional paper chromatography of the extract revealed the presence of more than 17 phenolic compounds, corresponding spots gave positive response toward $FeCl_3$ spray reagent, some of which appeared under UV light as dark purple spots, which turned orange or lemon yellow or reddish orange when fumed with ammonia vapor or when sprayed with Naturstuff spray reagent, a typical character of normal flavones or flavonol derivatives.

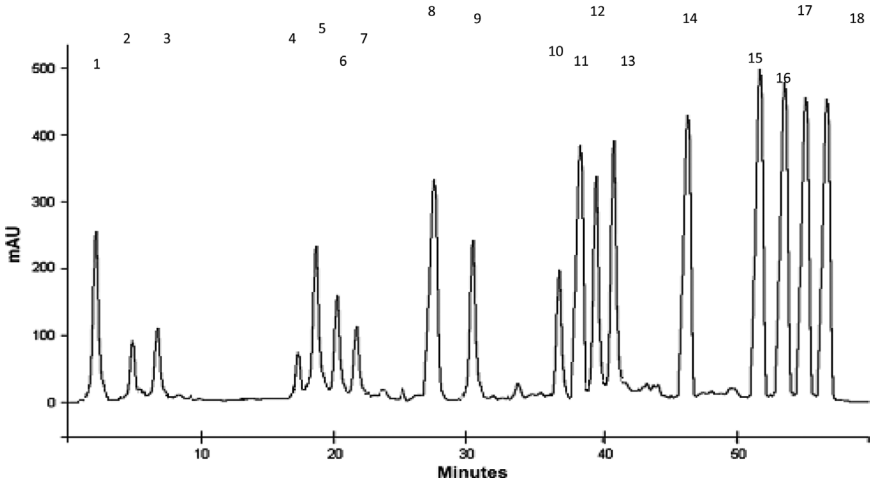
For the isolation of the phenolics contained in the extract, adsorption using HPLC was then engaged. A total of 18 major compounds for Roselle phenolics were identified by means of HPLC.

High-performance liquid chromatography

Further screenings of the phenolic compounds contained in the different methanolic extracts were achieved through RP-HPLC analysis of MRO. Phenolic content of the methanolic extracts of *H. sabdariffa* calyx are summarized in Table 2 and Fig. 1.

During the course of rearing period, the ranges of water temperature, dissolved oxygen, pH, and total ammonia were 27–29°C, 4.4–5.6 mg/l, 7.4–7.9, and 0.8–1.45 mg/l, respectively. All these parameters were within the acceptable range for Nile tilapia [34]. The results obtained in the present study revealed that supplementation of Nile tilapia diets with calyx at the tested levels (0.5; 1 and 1.5%) enhanced fish growth parameters compared with the control group, and the growth enhancement was more pronounced at 1% supplementation level. The improved fish growth may be due to the improved feed intake, which may possibly be due to the presence of *H. sabdariffa* used as a rich source of a mixture of essential fatty acids, including linoleic, linolenic, and arachidonic acids in the tested feed additive. These are required to prevent fatty acid deficiency diseases such as low growth [35] and are essential for growth [34,36], as Roselle calyx is used internally as a tonic for digestive and kidney function [37]. Moreover, an interesting characteristic of Roselle calyx is the presence of polyphenols, ascorbic acid [38], and red pigments (anthocyanin), which show

Figure 1



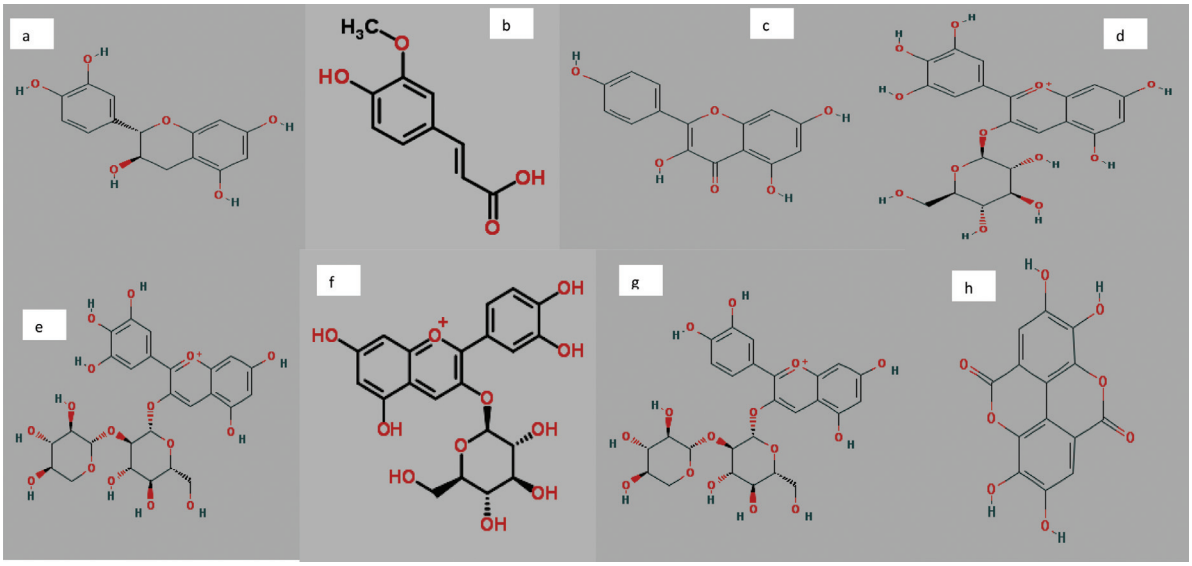
Typical high-performance liquid chromatogram of *Hibiscus sabdariffa* calyx phenolic profile showing the separation of a major chemical component.

Table 3 Growth performance of Nile tilapia fingerlings as affected with different levels of Roselle '*Hibiscus sabdariffa* L.' calyx for 12 weeks

	Control	Roselle calyx levels (%)		
		0.5	1.0	1.5
Initial weight (g)	5.44±0.01[a]	5.47±0.01[a]	5.46±0.01[a]	5.45±0.01[a]
Final weight (g)	18.33±0.17[c]	20.36±0.27[b]	24.22±0.39[a]	23.62±0.27[a]
Weight gain (g)	12.89±0.16[c]	14.89±0.28[b]	18.76±0.40[a]	18.17±0.26[a]
RBWG (%)	236.94±2.97[c]	272.21±5.48[b]	343.58±8.39[a]	333.39±4.21[a]
SGR (%/day)	1.44±0.01[c]	1.56±0.01[b]	1.77±0.02[a]	1.74±0.01[a]
Survival rate (%)	95.6±2.22[a]	95.6±2.22[a]	95.6±2.22[a]	95.6±2.22[a]

RBWG, relative body weight gain; SGR, specific growth rate. Mean having the same letter in the same row is not significantly different at $P<0.05$.

Figure 2



Chemical structure for the most abundant phenolic compounds identified in Roselle calyx using high-performance liquid chromatography. (a) Catechin; (b) ferulic acid; (c) kaempferol; (d) dephinidin-3-glucoside; (e) delphinidin-3-sambubioside; (f) cyanidin-3-glucoside; (g) cyanidin-3-sambubioside; (h) ellagic acid.

antioxidant activity [39]. These components could participate in fish metabolism, helping to improve health and growth. These results are in agreement with the results of Pérez *et al.*[40], who showed that increasing anthocynain in Roselle calyx/kg diet increased the growth rate, specific growth rate, and weight gain of goldfish (*Carassius auratus*) (Fig. 2 and Table 3).

In the present study, feed intake was higher for fish fed diets containing *H. sabdariffa* calyx at all levels, except fish fed dietary level of 1.0 and 1.5% *H. sabdariffa* calyx. This gradation, which was in the favor of fish on *H. sabdariffa* calyx, could be attributed to a high content of vitamins and minerals in *H. sabdariffa* calyx, which enhanced appetite [41] and it is evidenced by higher weight gain. Fish fed 1.0 and 1.5% *H. sabdariffa* calyx diet were the better supplemented level for feed conversion ratio (Table 4) in comparison with the control diet and other tested *H. sabdariffa* calyx levels. This is possibly because *H. sabdariffa* calyx increases the digestibility of feed [37], thereby making more nutrients available to the fish. Our present data also showed that feed efficiency ratio was significantly higher ($P<0.05$) for 1.5% *H. sabdariffa* calyx diet, whereas there was no significant difference in protein efficiency ratio, apparent protein utilization, and energy utilization between treatments (1.0 and 1.5%) of *H. sabdariffa* calyx. These results suggested that *H. sabdariffa* calyx supplementation did play a role in enhancing feed intake and feed utilization, with a subsequent enhancement of the fish body composition. Moreover, [40] showed that increasing anthocynain in *H. sabdariffa* calyx/kg diet, increased feed conversion rate of goldfish (*C. auratus*).

Table 4 Feed utilization of Nile tilapia fingerlings as affected with different levels of Roselle '*Hibiscus sabdariffa* L.' for 12 weeks

	Control	Roselle calyx levels (%)		
		0.5	1.0	1.5
Feed intake (g feed/fish)	23.44 ± 0.13^{c}	26.87 ± 0.53^{b}	29.57 ± 0.37^{a}	28.51 ± 0.52^{a}
Feed conversion ratio	1.82 ± 0.03^{a}	1.80 ± 0.01^{a}	1.57 ± 0.02^{b}	1.57 ± 0.01^{b}
Feed efficiency ratio	54.99 ± 1.00^{b}	55.41 ± 0.54^{b}	63.44 ± 0.82^{a}	63.73 ± 0.31^{a}
Protein efficiency ratio	1.97 ± 0.03^{b}	1.98 ± 0.01^{b}	2.23 ± 0.03^{a}	2.23 ± 0.01^{a}
Average protein utilization (%)	34.62 ± 0.56^{b}	35.90 ± 0.43^{b}	39.70 ± 0.46^{a}	39.83 ± 0.25^{a}
Efficiency utilization (%)	20.80 ± 0.35^{c}	22.06 ± 0.28^{b}	24.64 ± 0.28^{a}	24.84 ± 0.18^{a}

Mean having the same letter in the same row is not significantly different at $P < 0.05$.

Table 5 Proximate chemical analysis (% on dry matter basis) of whole body of Nile tilapia fingerlings as affected with different levels of Roselle '*Hibiscus sabdariffa* L.' for 12 weeks

	Control	Roselle calyx levels (%)		
		0.5	1.0	1.5
Moisture	73.41 ± 0.01^{a}	72.70 ± 0.07^{b}	72.72 ± 0.04^{b}	72.74 ± 0.05^{b}
Crude protein	60.15 ± 0.01^{b}	60.67 ± 0.03^{a}	60.71 ± 0.03^{a}	60.75 ± 0.02^{a}
Total lipids	20.32 ± 0.03^{c}	21.26 ± 0.05^{a}	$21.20 \pm 0.01^{a,b}$	21.13 ± 0.01^{b}
Ash	16.71 ± 0.03^{a}	16.60 ± 0.01^{a}	16.66 ± 0.01^{a}	16.64 ± 0.02^{a}

Mean having the same letter in the same row is not significantly different at $P < 0.05$.

After 12 weeks of feeding, the results showed no significant differences ($P > 0.05$) in moisture protein, lipids, and ash contents of whole tilapia body fed diets containing various levels of *H. sabdariffa* calyx. However, crude protein of fish bodies increased and total lipid was decreased significantly ($P < 0.05$) by increasing levels of *H. sabdariffa* calyx in the experimental diets. These results suggest that *H. sabdariffa* calyx supplementation plays a role in enhancing feed intake with subsequent effects on fish body composition.

Proximate chemical analysis of whole body of initial Nile tilapia fingerlings was as follows: moisture, 77.97; crude protein, 56; total lipids, 18.5; and ash, 24 (Table 5).

Hematological indices are a reflection of the effects of dietary treatments on the animal in terms of the type, quality, and amounts of the feed ingested and were available for the animal to meet its physiological, biochemical, and metabolic necessities, as regards hematological parameters analyzed (Hb, Ht, and RBCs) for Nile tilapia fed on *H. sabdariffa* calyx as natural feed additives. In the present study, there was a significant difference ($P < 0.05$) in Hb concentration, Ht, and RBC when all treatments were compared. In Hb, Ht, and RBC, there was a numerical increase as the level of *H. sabdariffa* L. calyx increased in concentration. This could be attributed to shift of water from the plasma to the muscle cells, thereby increasing the hemo concentration [42]. This probably suggest that a principle is in *H. sabdariffa* calyx that supports hemopoiesis since the value of RBC depends on those of Hb and Ht. This is in tandem with the work of Olusola [43], who found that the antioxidative potency of Roselle calyx extract resulted in a gradual increase in Hb, Ht, and RBC as the concentration increased. The positive physiological effect of this plant extract could be related to the presence of anthocyanins with a potent antioxidant activity. The above is also in consonance with the work of Unigwe [44], who reported that there was no significant difference ($P > 0.05$) but a numerical difference among Hb, Ht, and RBC as the concentration of Roselle calyx gradually increased (Table 6).

The concentration of total protein in blood plasma is used as a basic index for the health status of brood fish [45–48] as the measurement of serum or plasma albumin is of considerable diagnostic value in laboratory animals as it relates to general nutritional status, and the integrity of the vascular system and liver function. In our study, total protein, globulin, creatinine, and glucose increased significantly ($P < 0.05$) as a result of increased growth (Table 7).

These results suggest that high concentrations of total protein in fish serum were likely to be as a result of enhancement of the nonspecific immune response. These results are in agreement with those of Dorucu *et al.*[49], who reported a significant increase in serum

Table 6 Hemoglobin, red blood cell, and hematocrite of Nile tilapia fingerlings as affected with different levels of Roselle '*Hibiscus sabdariffa* L.' for 12 weeks

	Control	Roselle calyx levels (%)		
		0.5	1.0	1.5
Hemoglobin (g/dl)	5.30 ± 0.34^{c}	6.6 ± 0.34^{b}	9.3 ± 0.23^{a}	8.1 ± 0.11^{a}
Erythrocytes count (RBCs $\times 10^{6}$cells/mm^{3})	1.65 ± 0.04^{d}	1.84 ± 0.06^{c}	2.54 ± 0.03^{a}	2.35 ± 0.02^{b}
Haematocrite (%)	11.50 ± 0.11^{c}	13.0 ± 0.46^{b}	18.30 ± 0.51^{a}	17.80 ± 0.23^{a}

RBCs, red blood cells.
Mean having the same letter in the same row is not significantly different at $P < 0.05$.

Table 7 Biochemical blood plasma or serum changes of Nile tilapia fingerlings as affected with different levels of Roselle '*Hibiscus sabdariffa* L.' calyx for 12 weeks

		Roselle calyx levels (%)		
		0.5	1.0	1.5
Total lipids (g/dl)	8.26 ± 0.051^{a}	$8.14 \pm 0.08^{a,b}$	7.98 ± 0.02^{b}	7.20 ± 0.04^{c}
Total protein (g/dl)	1.58 ± 0.34^{d}	2.06 ± 0.69^{c}	2.27 ± 0.51^{b}	2.54 ± 0.69^{a}
Urea (mg/dl)	13.30 ± 0.51^{b}	16.0 ± 0.75^{a}	$14.50 \pm 0.46^{a,b}$	12.50 ± 0.63^{b}
Creatinine (mg/dl)	1.28 ± 0.11^{a}	1.48 ± 0.13^{a}	1.5 ± 0.10^{a}	1.48 ± 0.13^{a}
Glucose (mg/dl)	72.50 ± 0.40^{d}	78.0 ± 1.15^{c}	82.0 ± 1.15^{b}	87.10 ± 0.37^{a}
Albumin (g/dl)	0.69 ± 0.04^{b}	0.85 ± 0.04^{a}	0.86 ± 0.06^{a}	0.67 ± 0.01^{b}
Globulin (g/dl)	0.88 ± 0.01^{d}	1.19 ± 0.008^{c}	1.42 ± 0.01^{b}	1.87 ± 0.05^{a}
AST (GOT) (U/l)	52.0 ± 0.40^{c}	54.95 ± 1.35^{b}	63.20 ± 0.40^{a}	64.0 ± 0.75^{a}
ALT (GPT) (U/l)	48.0 ± 0.46^{a}	39.0 ± 0.69^{b}	38.50 ± 0.63^{b}	39.0 ± 0.23^{b}

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.

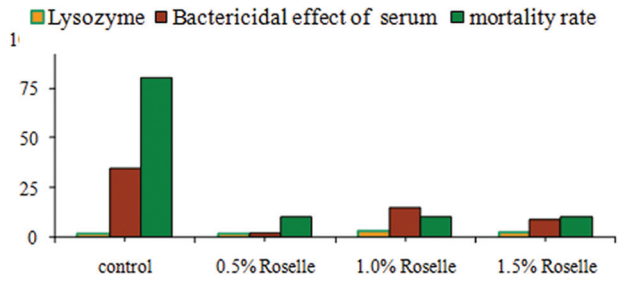
protein and total immunoglobulin levels in rainbow trout fed with basal diet incorporated with 1, 2.5, and 5% annual flowering plant, *Nigella sativa*, compared with the control group. Similarly, increases in total protein, albumin, and globulin levels in common carp fed with different doses of extracts of a herbal mixture were recorded [50].

AST, ALT, urea, serum albumin, and total lipid were decreased at 1.5% *H. sabdariffa*. This decrease in AST and ALT suggests that the administration of *H. sabdariffa* has a protective effect on the level of circulatory liver marker enzymes and hence liver damage. This finding is consistent with results of Lin *et al.*[51], who showed that various extracts of calyces of *H. sabdariffa* L., including *H. sabdariffa* extract, *H. sabdariffa* anthocyanins, and *H. sabdariffa* polyphenol rich extracts, have been reported to exhibit activities against atherosclerosis, liver disease, and other metabolic syndromes. Hibiscus flowers contain gossypetin, glucoside, bibiscin, hibiscus anthocyanin, and hibiscus protocatechuic acid and have the following effects: choleretic and diuretic functions, decreasing blood pressure, reducing the viscosity of the blood, and stimulating intestinal peristalsis [52,53]. Thus, the dried flowers of *H. sabdariffa* are a functional natural product with a chemopreventive capacity.

The mortality rate of fish fed with *H. sabdariffa* calyx diets and challenged by *A. hydrophila* for 10 days was 10%, whereas it was high in fish fed the control diet (80%). These results indicate that *H. sabdariffa* calyx had high antibacterial effect against pathogenic *A. hydrophila*, due to the presence of polyphenols, ascorbic acid [38], and red pigments, which show antioxidant capacity and may be related to essential oils that contain substituted phenols; eugenol exhibits strong antibacterial antioxidant effects [54]. These results are in agreement with those of Faraji and Tarkhani [55] and Tseng *et al.*[56], who indicated that Roselle calyces have positive health effects.

At the end of the experimental period, lysozyme level increased in the serum of tilapia that was fed with diet containing *H. sabdariffa* calyx, whereas the highest value was recorded at 1.0% *H. sabdariffa* calyx diet compared with fish group fed with control diet. Lysozyme appeared to be an important component of the immune system of fish, as any form of pathogen challenge or environmental stress factor resulted in a subsequent change in lysozyme

Figure 3



Effect of *H. sabdariffa* calyx on lysozyme, bactericidal and mortality rate.

activity [57]. Roselle has antimicrobial activities due to its phenolic compounds [58]. These results suggest that the *H. sabdariffa* supplementation could increase the nonspecific immune system of Nile tilapia resulting in fish resistance to *A. hydrophila* infection.

SBT is a mechanism noted for the killing and clearing of pathogenic organisms in fish [59]. *A. hydrophilla* was used as a model to examine the effectiveness of both supplements to kill the bacterial infection. The lowest number of bacterial colonies indicated the efficiency of immune cells in serum to kill the pathogen. The results of the present study revealed that SBT of fish fed with *H. sabdariffa* (0.5, 1.0, and 1.5%) against *A. hydrophila* were higher than that in control. The viable bacterial counts were significantly lower in all treatments groups [2, 15, and $9\,(\text{cfu/ml}) \times 10^6$, respectively, when compared with the control group, $35 \times 10^6\,(\text{cfu/ml})$]. The superiority of 1.0% may lead us to believe that this level provides fish with proper concentration of effective compounds that have antibacterial effects. These results related to the fact that in the calyxes of Roselle certain amounts of delphinidin-3-monoglucoside and cyanidin-3-monoglucoside, which constitute the anthocyanin, are also present [60]. There are indications that extracts from the red calyxes of Roselle contain antioxidant principles [61–64] (Fig. 3).

Conclusion

Results obtained in the present study showed that the potential of using *H. sabdariffa* calyx enhances immune and health status and improves disease resistance in Nile tilapia, thereby improving growth performance.

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Conflicts of interest

There are no conflicts of interest.

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