



# Effect of dietary seaweed extract supplementation on growth, feed utilization, hematological indices, and non-specific immunity of Nile Tilapia, *Oreochromis niloticus* challenged with *Aeromonas hydrophila*

Mohamed Ashour<sup>1</sup> · Mohamed M. Mabrouk<sup>2</sup> · Hala F. Ayoub<sup>3</sup> · Mohamed M.M. El-Feky<sup>1</sup> · Sharawy Z. Zaki<sup>1</sup> · Seyed Hossein Hoseinifar<sup>4</sup> · Waldemar Rossi Jr<sup>5</sup> · Hien Van Doan<sup>4</sup> · Ehab El-Haroun<sup>6</sup>  · Ashraf M.A-S. Goda<sup>1</sup>

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## Abstract

Seaweeds have increasing importance because of their bioactive compounds and their potential application in different industries. Liquid seaweed extract are commercially used as an agricultural biofertilizer. The current work investigated the potential of a commercial liquid seaweed extract, named TrueAlgaeMax (TAM), as a diet feed additive for Nile tilapia *Oreochromis niloticus*. Five diets (D<sub>0</sub>, D<sub>0.5</sub>, D<sub>1</sub>, D<sub>1.5</sub>, and D<sub>2</sub>) were supplemented with different TAM concentrations (0, 0.5, 1, 1.5, and 2%, respectively) to investigate the growth performance, feed utilization, and non-specific immunity of *O. niloticus* challenged with *Aeromonas hydrophila*. Healthy mono-sex *O. niloticus* fingerlings were randomly stocked for 70 days in net enclosures (3 m × 6 m × 1 m) at a density of 20 fish m<sup>-3</sup> with an average initial body weight of 50 g fingerling<sup>-1</sup> (three groups for each treatment with a total of 1080 fish per treatment). Diet D<sub>2</sub> represented the highest weight gain (WG) value (161.29 g), average daily weight gain (ADWG) value (2.30 g day<sup>-1</sup>), and specific growth rate (SGR) value (2.40% day<sup>-1</sup>) and achieved the best feed conversion ratio (FCR) value (1.31). In addition, D<sub>2</sub> achieved the highest serum protein (5.47 g dL<sup>-1</sup>), lysozyme (3.75 μg mL<sup>-1</sup>), and respiratory burst activity (1.89 mg mL<sup>-1</sup>). Fish mortality decreased by increasing TAM levels. The GC-Mass data of TAM showed nine main phytochemical compounds belonging to seven different important bioactive groups reported as immunity enhancer and growth promoting and showed antioxidant and antimicrobial properties. The results recommended that increasing the levels of TAM up to 2% as feed additive for *O. niloticus* diet strongly enhances growth performance, feed utilization, and non-specific immunity of Nile tilapia *O. niloticus* challenged with *A. hydrophila*. Considering seaweed additions (dry weight or extract) as aquaculture feed additive is of importance.

**Keywords** Liquid feed additive · TAM · FAMES · Oleic acids · Milbemycin · 5-Silaspiro · Growth performance · Non-specific immunity

✉ Ehab El-Haroun  
elharoun@gmail.com

- <sup>1</sup> Aquaculture Division, National Institute of Oceanography and Fisheries, Alexandria, Egypt
- <sup>2</sup> Animal and Fish Production Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt
- <sup>3</sup> Department of Fish health and Management, Central laboratory for Aquaculture Research, Agricultural Research center (ARC), Abbassa, sharqia, Egypt
- <sup>4</sup> Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
- <sup>5</sup> School of Aquaculture and Aquatic Sciences, Kentucky State University, Frankfort, KY, USA
- <sup>6</sup> Fish Nutrition Research Laboratory, Animal Production Department, Faculty of Agriculture, Cairo University, Cairo, Egypt

## Introduction

Seaweeds are considered important marine ecosystem components. Recently, seaweeds are becoming of increasing importance because of their bioactive compounds and their applications (El-Shenody et al. 2019; Davies et al. 2020; Bowyer et al. 2020; Hosseinifer et al. 2020). According to FAO (2018), the total global production of seaweeds in 2015 is 30.4 million tonnes and the culture and wild capture sector was 29.4 and 1.0 million tonnes, respectively. Today, the annually world seaweed industry is more than US\$ 6 billion. In 2016, the major farmed seaweeds producers were China (47.9%), Indonesia (38.7%), Philippines (4.7%), and Republic of Korea (4.5%), while major wild seaweeds producers were Chile, China, and Norway (FAO 2018). To

achieve a successful commercial and biotechnological potential of algae, screening of native species must be considered. The Egyptian coast has a wide diversity of nutritious seaweeds all the year around (Khairy and El-Sheikh 2015; El-Shenody et al. 2019). The chlorophyte *Ulva lactuca* and rhodophytes *Pterocladia capillacea* and *Jania rubens* are among the dominant seaweed species in Egypt. The biochemical composition, antioxidant activity, and mineral composition of these three species was previously evaluated by Khairy and El-Shafay (2013) and Khairy and El-Sheikh (2015).

Liquid seaweed extract are commonly used as commercial agricultural biostimulants. Various bioactive compounds such as alginates, cytokinins, auxins, polyamines, abscisic acid, gibberellins, and brassinosteroids have been observed in various commercial seaweed extracts (Kannan et al. 2016).

Extensive studies have been conducted to evaluate the effect of whole seaweed species as additional feed additive to enhance growth performance, nutrients composition, and feed utilization of many fish species like Nile tilapia (Meurer et al. 2009; Hussein 2017), rabbit fish (Abdel-Aziz and Ragab 2017), and red tilapia (El-Tawil 2010) without any negative effect on feed efficiency or survival rate. The increase of aquaculture development has led to increased demand for improved diet and improved aquaculture feed additives. The use of seaweed as an aquaculture feed additive has been reported by many researchers. Recently, the use of bioactive materials extracted from seaweed as an aquaculture feed additive has demonstrated positive responses on growth performance, feed utilization, enhance serum biochemical composition, immune responses, hematological parameters, and disease resistance for many different cultured aquaculture species (Akbarly et al. 2018; Akbarly and Aminikhoei 2018; Ardo et al. 2008). For example, the methanolic extract of the marine seaweed *Caulerpa scalpelliformis* has the potential to be developed into an immunostimulant for *O. niloticus* (Yengkhom et al. 2016). Similarly, polysaccharides extracted from marine seaweeds have been widely studied for their immune-stimulatory effects and disease resistance in *O. niloticus* (Doan et al. 2017; Yengkhom et al. 2018; Hosseini et al. 2020), gray mullet *Mugil cephalus* (Akbarly and Aminikhoei 2018), rainbow trout *Oncorhynchus mykiss* (Zeraatpisheh et al. 2018), Asian sea bass *Lates calcarifer* (Yangthong et al. 2016), black tiger shrimp *Penaeus monodon* (Chotigeat et al. 2004), and *Fenneropenaeus chinensis* (Huang et al. 2006).

The current study aims to investigate the effect of different levels of liquid feed additive (TrueAlgaeMax (TAM)) prepared from seaweed extract on growth performance, feed utilization, and non-specific immunity for Nile tilapia

*Oreochromis niloticus*, challenged with *Aeromonas hydrophila*.

## Materials and methods

### Experimental fish and culture technique

All male Nile tilapia, *Oreochromis niloticus* with an average initial body weight of  $50.0 \pm 0.2$  g were obtained from a private Tilapia incubation, Port Said Governorate, Egypt. The on-growing fish were stocked into a rectangular earthen pond at a private fish farm located at Shader Azzam, East of Tafia, South of Port Said, Port Said Governorate with total area 8400 m<sup>2</sup>. The pond was installed with 15 equal net enclosures (each of 18 m<sup>3</sup>, 3 m × 6 m × 1 m). Each net enclosure was stocked with 20 fish m<sup>-3</sup> (a total of 360 fish per treatment). Three replicate net enclosures were randomly assigned to each treatment. The net enclosures were suspended on the pond water surface by direct attachment to wooden piers, set on wooden stakes, and attached to wooden structures. The nets were cleaned regularly during experimental period. The total water pond turnover rate was 10% pond<sup>-1</sup> day<sup>-1</sup>. Fish were held under natural light (12:12 h light:dark schedule).

Prior to the experiment, the earthen pond was prepared well by de-watering, cleaning well, and filling with water (on day 2) and addition of quicklime (on day 3) by spreading methods at the rate of 100 kg per 4200 m<sup>2</sup>. After 1 week of lime addition, urea and Triple super phosphate (TSP (5 kg pond<sup>-1</sup> of each) were mixed and dissolved in plastic bucket for 10–12 h then applied to the pond. After 21 days of fertilizers addition, *O. niloticus* mono-sex fingerlings were stocked in net enclosures.

The main source of pond water was the irrigation water of Al Salam Chanel. Water temperature, dissolved oxygen, pH, and ammonia, nitrate and nitrite were monitored during the trial, to maintain water quality at optimum range for Nile tilapia. Water temperature was recorded daily at 13.00 h using a mercury thermometer suspended at 30-cm depth. Dissolved oxygen (DO) was measured at 05.00 h using YSI model 56 oxygen meter (Yellow Springs Instrument, USA) and pH at 09.00 h by using a pH meter (Orion, USA). Ammonia nitrate and nitrite were measured three times a week according to APHA (2005).

### Characteristics of seaweed liquid extract (TAM)

#### Sampling

Seaweed species used in the manufacturing of TAM (seaweed liquid extract product) were *Ulva lactuca*, *Jania rubens*, and *Pterocladia capillacea* collected during the summer season of 2017 from Boughaz El-Maadya, Abu-Qir Bay (31° 16' 16.0" N, 30° 10' 28.0" E), Alexandria, Egypt. After seaweed

harvesting, the extraneous matter, epiphytes, and wastes were removed. Samples were washed to remove sand and salt particles, air dried, and then powdered and kept at room temperature in plastic bag for further analysis (Ashour et al. 2020).

**TAM proximate and phytochemical analysis**

TAM is a commercial product and submitted as a patent (Ashour 2019). The TAM contents of organic matter, polysaccharides, total dissolved solids, major elements (N, P, and K), minor element (copper, iron, magnesium, zinc, and manganese), and heavy metals (cadmium, chromium, lead, nickel, and arsenic), as well as, color, odor, density, and pH were determined according to APHA (2005). To determine the phytochemical constituent of crude TAM, GC-Mass Spectrophotometry analysis was conducted according to Elshobary et al. (2020). The identification of unknown compounds was performed based on comparing the obtained mass spectra with those available in NIST library.

**Experimental diets**

Five experimental diets were formulated to be isonitrogenous, 30% crude protein (CP) and isocaloric, 15.45 MJ kg<sup>-1</sup> digestible energy (DE). All diets were identical except for the variation in seaweed extract (TAM) levels. The basal experimental diet (control diet) had no TAM added (D<sub>0</sub>). Diets 2–5 each contained TAM at levels of 0.5 (D<sub>0.5</sub>), 1 (D<sub>1</sub>), 1.5 (D<sub>1.5</sub>), and

2% (D<sub>2</sub>), respectively. The proximate chemical composition of the basal experimental diet is presented in Table 1. Gross energy (GE) contents of diets were calculated according to gross caloric values of Jobling (1983) using the values of 23.6, 39.5, and 17.2 kJ g<sup>-1</sup> for crude protein, crude fat, and total carbohydrate, respectively. Proximate analysis of feed diet ingredients is presented in Table 2.

The diets were processed by blending the dry ingredients into a homogenous mixture. Pellets of 2 mm were made in Sprout-Waldron Laboratory Pellet Mill (CPM, California Pellet Mill Co., USA). The pelleting temperature did not exceed 40 °C. Seaweed extract (TAM) was dissolved in distilled water and then sprayed on the diet surface according to methods of Mehrabi et al. (2012). The same adequate volume of distilled water was sprayed on the control diet (D<sub>1</sub>) without TAM. All diets were drying at 40 °C for 48 h (moisture content of about 10%), then 5 mL kg<sup>-1</sup> diet of sunflower oil was sprayed over the basal diet to cover the aqueous extract (Zeraatpisheh et al. 2018) and finally air dried for 4 h. All diets were packed in cellophane bags and cooled at 4 °C prior to use. During the 70-day experimental period, all fish were fed their respective diets at a level of 3% of body weight. The daily ration was divided into three equal amounts and offered three times a day (09:00, 12:00, and 16:00 hours). Random samples of at least 75% of fish from each replicate net enclosure were weighed biweekly, and the amount of daily allowance was adjusted accordingly.

**Growth indices**

Mean final body weight (FBW) of each experimental treatment was determined by dividing total fish weight in each net enclosure by the number of fish. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), protein

**Table 1** Diet formulation and proximate analysis (g kg<sup>-1</sup>)

Diet ingredients (%)	
Fish meal	26
Soybean meal	18
Yellow corn	35
Wheat bran	8
Linseed oil	6
Vit. mix	2
Min mix	2
Carboxyl methyl cellulose	3
Total	100
Chemical composition (g kg <sup>-1</sup> )	
CP %	298
EE %	96
CF %	33.5
Ash %	98
Gross energy <sup>a</sup> (GE; MJ kg <sup>-1</sup> )	20.90

DM %, dry matter content; CP %, crude protein; EE %, ether extract; CF %, crude fiber; NFE %, nitrogen-free extract

<sup>a</sup> Calculated using gross calorific values of 23.63, 39.52, and 17.15 kJ g<sup>-1</sup>

**Table 2** Proximate analysis of feed ingredients used in the experimental diet (g kg<sup>-1</sup> of DM basis)

Items	Fish meal	Soybean meal	Yellow corn	Wheat bran
DM %	920	900	880	890
CP %	700	440	850	164
EE %	85	11	36	40
CF %	6	73	23	99
Ash %	114	63	13	53
NFE %	95	413	843	644
GE (kcal kg <sup>-1</sup> )	5162	4534	4284	4276
DE (kcal kg <sup>-1</sup> )	3871	3400	3213	3207

DM %, dry matter content; CP %, crude protein; EE %, ether extract; CF %, crude fiber; NFE %, nitrogen-free extract; GE, gross energy; DE, digestible energy (kcal kg<sup>-1</sup>)

intake (PI), fat retention (FR), and energy retention (ER) were calculated using the following equations:

$$\text{WG} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

$$\text{SGR} = (\ln \text{FBW} - \ln \text{IBW}) / t \times 100$$

where: *FBW* is final body weight (g); *IBW* is initial body weight (g); and *t* = time in days

$$\text{FCR} = \text{Feed intake (g)} / \text{weight gain (g)}$$

$$\text{PER} = \text{weight gain (g)} / \text{protein intake (g)}$$

$$\text{PPV} = (\text{protein gain (g)} / \text{protein intake (g)}) \times 100$$

$$\text{FR} = (\text{fat gain (g)} / \text{fat intake (g)}) \times 100$$

$$\text{ER} = (\text{energy gain (kJ)} / \text{energy intake (kJ)}) \times 100.$$

$$\begin{aligned} \text{Feed Efficiency Ratio (FER)} \\ = \text{Body weight gain (g)} / \text{Feed Intake (g)}; \end{aligned}$$

$$\text{Protein Intake (PI)} = \text{Feed Intake (g)} / \text{Protein\%}.$$

## Analytical methods

At the beginning of the trial, a random pooled sample of 20 fish was collected and killed to determine the initial whole-body proximate composition. At the termination of the feeding trial, five fish were randomly selected from each net enclosure, killed, and homogenized in a blender to determine the final whole-body proximate composition. The fish were pooled for each net enclosure, oven dried, ground, and stored at  $-20\text{ }^{\circ}\text{C}$  for subsequent analysis. The chemical composition of fish and diet samples were determined according to the procedures of AOAC (2003). Dry matter was determined after drying the samples in an oven ( $105\text{ }^{\circ}\text{C}$ ) for 24 h. Ash was incinerated at  $550\text{ }^{\circ}\text{C}$  for 12 h. Crude protein was determined by micro-Kjeldhal method,  $N\% \times 6.25$  (using Kjeltech

Autoanalyzer, Model 1030, Tecator, Sweden) and crude fat by Soxhlet extraction with diethyl ether ( $40\text{--}60\text{ }^{\circ}\text{C}$ ). Nitrogen-free extract (NFE) was calculated by subtracting CF, CP, and EE from total organic matter content. Gross energy (GE) contents of diets were calculated according to gross caloric values of Jobling (1983) using the values of 23.6, 39.5, and  $17.2\text{ kJ g}^{-1}$  for crude protein, crude fat, and total carbohydrate, respectively. The DE was estimated by applying the coefficient of 70% from determined GE values according to Hephher et al. (1983).

## Blood samples and analysis

At the end of the experiment, blood samples were collected via the caudal blood vessels randomly chosen from five fish from each net enclosure using needles previously rinsed in heparin ( $15\text{ unit mL}^{-1}$ ). The other blood samples were centrifuged at  $585 \times g$  for 20 min to obtain serum. The serum samples were stored at  $-80\text{ }^{\circ}\text{C}$  until use. Total serum protein and albumin were determined according to Lowry et al. (1951) and Wotton and Freeman (1982), respectively. Globulin was calculated by subtracting albumin level from total protein. Glucose and triglycerides was determined according to Henry (1964) and MGowan et al. (1983), respectively. Besides, the level of serum glutamic pyruvate transaminase (GPT) was measured using kits of Biomerieux, France, according to the producer instructions.

## Non-specific immunity assay

### Serum lysozyme activity

Lysozyme activity was measured using photoelectric colorimeter with attachment for turbidity measurement. A series of dilution was prepared by diluting the standard lysozyme from hen egg-white (Fluka, Switzerland) and mixed with *Micrococcus lysodeikticus* (ATCC No. 1698 Sigma) suspension for establishing the calibration curve. Ten microliters of standard solution or serum were added to 200  $\mu\text{L}$  of *Micrococcus* suspension (35 mg of *Micrococcus* dry powder per 95 mL of 1/15 M phosphate buffer and 5.0 mL of NaCl solution). The changes in the extinction were measured using spectrophotometer at 546 nm by measuring the extinction immediately after adding the solution which contained the lysozyme (start of the reaction) and after a 20-min incubation of the preparation under investigation at  $40\text{ }^{\circ}\text{C}$  (end of the reaction). The lysozyme activity is determined using the calibration curve and the extinction measured (Schäperclaus 1992).

## Respiratory burst assay

Respiratory burst activity was assayed by the reduction of nitro blue tetrazolium (NBT) intracellular super peroxide radicals, 0.1 mL blood was placed into micro-titer plate then equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature, 0.1 mL of NBT blood cell suspension was taken and added to a glass tube containing 1 mL *N,N*-dimethyl formamide and centrifuged for 5 min at 500×*g*, and the supernatant was read at 620 nm (Siwicki et al. 1994).

## Challenge with *Aeromonas hydrophila*

To investigate the challenge test of TAM used in the current experiment, immune-stimulant intra-peritoneal injection of live *A. hydrophila* was conducted. *A. hydrophila* bacterium strain was isolated, identified, and prepared by the Fish Health and Diseases Department, Central Lab. for Aquaculture Research, Abbassa, Egypt. On 70 days after start feeding with TAM, 30 fish from each experimental group were injected intra-peritoneally with 0.1 mL of live *A. hydrophila* ( $1 \times 10^8$  CFU mL<sup>-1</sup>) which had previously been isolated from moribund fish and identified using the VITEK 2-C15 automated system for identification of bacteria (BioMerieux Inc., France) according to manufacturer's instructions. The mortality was monitored for 10 days. The fish were checked regularly for overt signs of disease including behavioral abnormalities. Mortality was noted in all the groups daily, and relative percent survival (RPS) was estimated (Amend 1981) using formula;

$$\text{RPS} = 1 - (\% \text{mortality in challenged fish} / \% \text{mortality in control fish}) \times 100$$

## Statistical analysis

Results were statistically analyzed by one way ANOVA using SPSS (IBM, v. 20). Least significant difference multiple range test (LSD) was used to compare differences between treatment means when significant *F* values were observed, at  $P \leq 0.05$  levels. All percentage data were arc-sin transformed prior to analysis (Zar 1984). However, data are presented untransformed to facilitate comparisons. The relationship between hematological indices was tested using simple correlation analysis.

## Results

No mortality was recorded for fish fed the diets with or without TAM supplementation during the 70 days of the experimental period. All the water quality parameters were shown within the acceptable range for Nile tilapia (Wilson 1991). Water temperature ranged from 26.6 to 28.7 °C, dissolved oxygen (DO) from 5.60 to 6.45 mg L<sup>-1</sup>, pH from 7.54 to 7.72, ammonia (NH<sub>3</sub>) from 0.16 to 0.34 mg L<sup>-1</sup>, nitrate from 0.16 to 0.34 mg L<sup>-1</sup>, and nitrite from 0.08 to 0.50 mg L<sup>-1</sup>.

## TAM proximate and phytochemical analysis

Physical investigations showed that TAM has dark brown color, seaweed odor, density of 1.2, and pH 9–9.5 (Table 3). Biochemical analyses of total polysaccharides, total organic matter, and total dissolved solids, as well as major and minor elements are presented in Table 3. Heavy metal data analysis showed that cadmium, chromium, lead, and nickel were not

**Table 3** Physical, chemical, and biochemical analyses of TAM

Item	Value
Physical analyses	
Color	Dark brown
Odor	Seaweed
Density	1.20
pH	9–9.5
Biochemical analyses (% DM)	
Total polysaccharides	15
Total organic matter	23.2
Total dissolved solids	2.6
Chemical analyses	
Major elements	
Potassium (%)	12
Phosphorus (%)	2.4
Total nitrogen (ppm)	1400
Minor elements (ppm)	
Copper	0.39
Iron	16.18
Magnesium	19.72
Zinc	1.19
Manganese	3.72
Heavy metals (ppm)	
Cadmium	0.00
Chromium	0.00
Lead	0.00
Nickel	0.00
Arsenic	0.55



**Table 4** Growth performance and feed utilization of *O. niloticus* fed on diets supplemented with different levels of TAM

Diets	Growth performance					Feed utilization						
	IW (g)	FW (g)	WG (g)	AWG (g day <sup>-1</sup> )	SGR (% day <sup>-1</sup> )	FI (g)	PI (g)	FCR feed: gain	FER	PER		
D <sub>0</sub> (control)	49.98 ± 0.10a	186.17 ± 0.44c	136.18 ± 0.24c	1.94 ± 0.03c	2.20 ± 0.0b	205.7 ± 0.4b	61.7 ± 0.1b	1.51 ± 0.01ab	0.66 ± 0.00cd	2.21 ± 0.01c		
D <sub>0.5</sub>	49.99 ± 0.08a	187.89 ± 0.92c	137.90 ± 0.49c	1.97 ± 0.00c	2.20 ± 0.0b	213.5 ± 1.6ab	64.0 ± 0.4ab	1.55 ± 0.01a	0.64 ± 0.00d	2.15 ± 0.01c		
D <sub>1</sub>	49.99 ± 0.04a	199.67 ± 7.26b	149.55 ± 4.21b	2.13 ± 0.06b	2.33 ± 0.0a	216.2 ± 2.0a	64.8 ± 0.6a	1.45 ± 0.05bc	0.69 ± 0.02bc	2.31 ± 0.08bc		
D <sub>1.5</sub>	49.95 ± 0.09a	208.94 ± 6.11a	158.98 ± 3.57a	2.27 ± 0.05a	2.37 ± 0.6a	217.6 ± 0.6a	65.2 ± 0.2a	1.37 ± 0.04cd	0.73 ± 0.02ab	2.43 ± 0.06ab		
D <sub>2</sub>	49.98 ± 0.08a	211.28 ± 5.44a	161.29 ± 3.11a	2.30 ± 0.04a	2.40 ± 0.0a	211.1 ± 5.6ab	63.3 ± 1.7ab	1.31 ± 0.02d	0.76 ± 0.01a	2.55 ± 0.04a		

Data were represented as means ± SD. Different lowercase letters in each column indicate significant differences ( $P < 0.05$ )

D<sub>0</sub>, diet supplemented with 0% TAM; D<sub>0.5</sub>, diet supplemented with 0.5% TAM; D<sub>1</sub>, diet supplemented with 1% TAM; D<sub>1.5</sub>, diet supplemented with 1.5% TAM; D<sub>2</sub>, diet supplemented with 2% TAM; IW, initial weight; FW, final weight; WG, weight gain; ADWG, average daily gain; SGR, specific growth rate; FI, feed intake; PI, protein intake; FCR, feed conversion ratio; FER, feed efficiency ratio; PER, protein efficiency ratio

detected, while arsenic achieved 0.55 ppm. The GC-MS analysis of TAM showed nine main phytochemical compounds belonging to nine retention times depending on the highest probability (Fig. 1). These different bioactive phytochemical compounds belong to seven important groups. Methylated fatty acids (fatty acid methyl esters) was the major group (three RT with 80.23% of total peak area) and consist of three methylated FA; tridecanoic acid, methyl ester (50.01%); 9,12-octadecadienoic acid, methyl ester, (E,E)- (16.06%); and  $\gamma$ -linolenic acid methyl ester (14.16%). The other phytochemical compounds are phytol (diterpene alcohol), milbemycin oxime (macrocyclic lactones), nonadecane (alkane hydrocarbon), oleic acid (natural fatty acid), and rhodopin (carotenoid) with peak area percentages of 6.31, 4.55, 3.64, 2.76, and 0.78%, respectively. Moreover, 5-silaspiro[4.4]nona-1,3,6,8-tetraene,3,8-bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl was detected with a peak area of 1.85%. Mass spectra and retention times of the nine phytochemical compounds identified with NIST library are shown in Fig. 2.

### Growth performance and feed utilization

All fish fed TAM-supplemented diets showed higher growth performance and feed efficiency than the control diet (Table 4), suggesting that the addition of TAM enhanced the growth performance. Significantly higher growth performance and feed utilizations were observed for fish fed either D<sub>1.5</sub> or D<sub>2</sub> diets compared with the lower values observed for fish fed either control diet D<sub>0</sub> or D<sub>0.5</sub> diet. Nile tilapia fed the diets D<sub>1.5</sub> and D<sub>2</sub> had significantly higher FW, WG, and SGR. Fish fed the D<sub>1.5</sub> and D<sub>1</sub> diets showed a significantly higher FI and PI respectively followed by D<sub>0.5</sub> and D<sub>2</sub>, whereas the lowest FI and PI was observed in fish fed the control diet D<sub>0</sub> without seaweed extract supplementation. Fish fed the diets D<sub>0.5</sub>, D<sub>0</sub>, D<sub>1</sub>, and D<sub>1.5</sub> had the lowest FCR in decreasing order, while the best FCR values were achieved by fish fed either D<sub>2</sub> or D<sub>1.5</sub>.

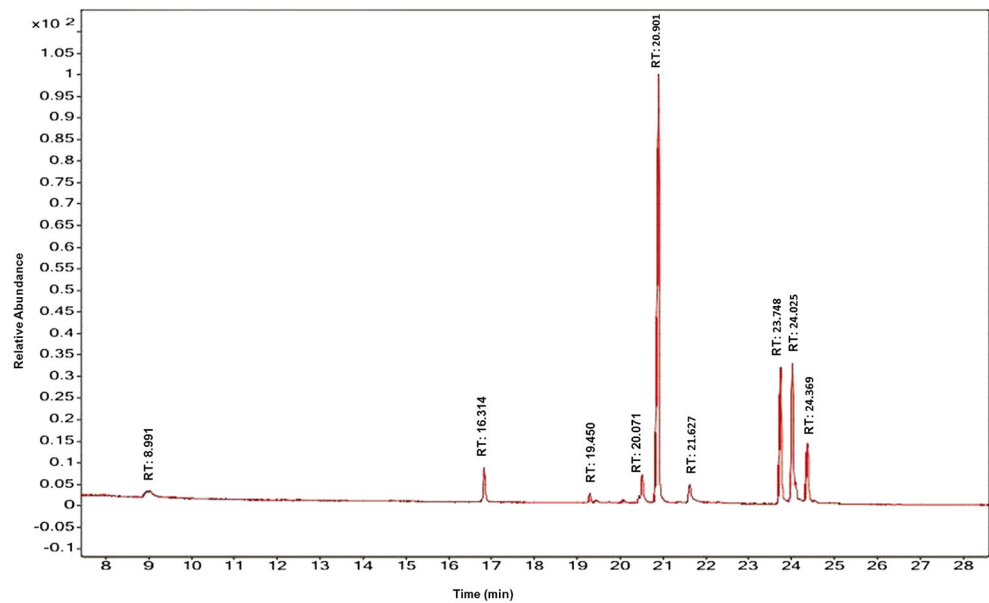
### Fish biochemical analysis

The biochemical composition (CP, EE, and ash) of *O. niloticus* was determined (% of dry matter) at the end of the feeding experiment, as summarized in Table 5. The highest ( $P < 0.05$ ) CP and EE values were observed with the D<sub>2</sub> diet followed by D<sub>1.5</sub> and D<sub>1</sub> while the lowest ( $P < 0.05$ ) protein was achieved by D<sub>0.5</sub> and D<sub>0</sub> (Table 6). The results showed that D<sub>0.5</sub> and D<sub>1</sub> achieved the highest ( $P < 0.05$ ) ash content followed by D<sub>1.5</sub> and D<sub>2</sub>, while the lowest ( $P < 0.05$ ) ash content was achieved by D<sub>2</sub> (Table 5).

### Serum biochemical parameters

Diets supplemented with different levels of TAM resulted in higher ( $P \leq 0.05$ ) total serum protein, albumin, globulin, and

**Fig. 1** GC-MS chromatogram of crude TAM



triglycerides values (Table 6), while no significant differences were obtained for glucose and GPT (Table 6). The highest significant ( $P < 0.05$ ) total protein, albumin, and globulin were observed with  $D_2$ ,  $D_{1.5}$ ,  $D_1$ , and  $D_{0.5}$ , while the lowest values were observed with the control diet (Table 6). On the other hand, the highest significance ( $P < 0.05$ ) of triglyceride was observed for the control diet, followed by  $D_1$ ,  $D_{1.5}$ , and  $D_2$  and the lowest triglyceride ( $P < 0.05$ ) was observed with  $D_{2.5}$  (Table 6).

### Serum lysozyme and respiratory burst activity

Table 7 shows significant ( $P \leq 0.05$ ) increases in lysozyme and respiratory burst activity, in the fish fed diets containing increasing levels of TAM compared with the control diet.

### Challenge with *Aeromonas hydrophila*

The cumulative mortality of 10 days post-challenge with *A. hydrophila* was highest in fish fed the control diet  $D_0$ . The lowest mortality was observed in fish fed  $D_2$ , followed by  $D_{1.5}$ ,  $D_1$ , and  $D_{0.5}$  (Table 7). The highest RPS was obtained with diet  $D_2$ , followed by  $D_{1.5}$ ,  $D_1$ , and  $D_{0.5}$ , whereas the lowest RPS was observed with the control diet.

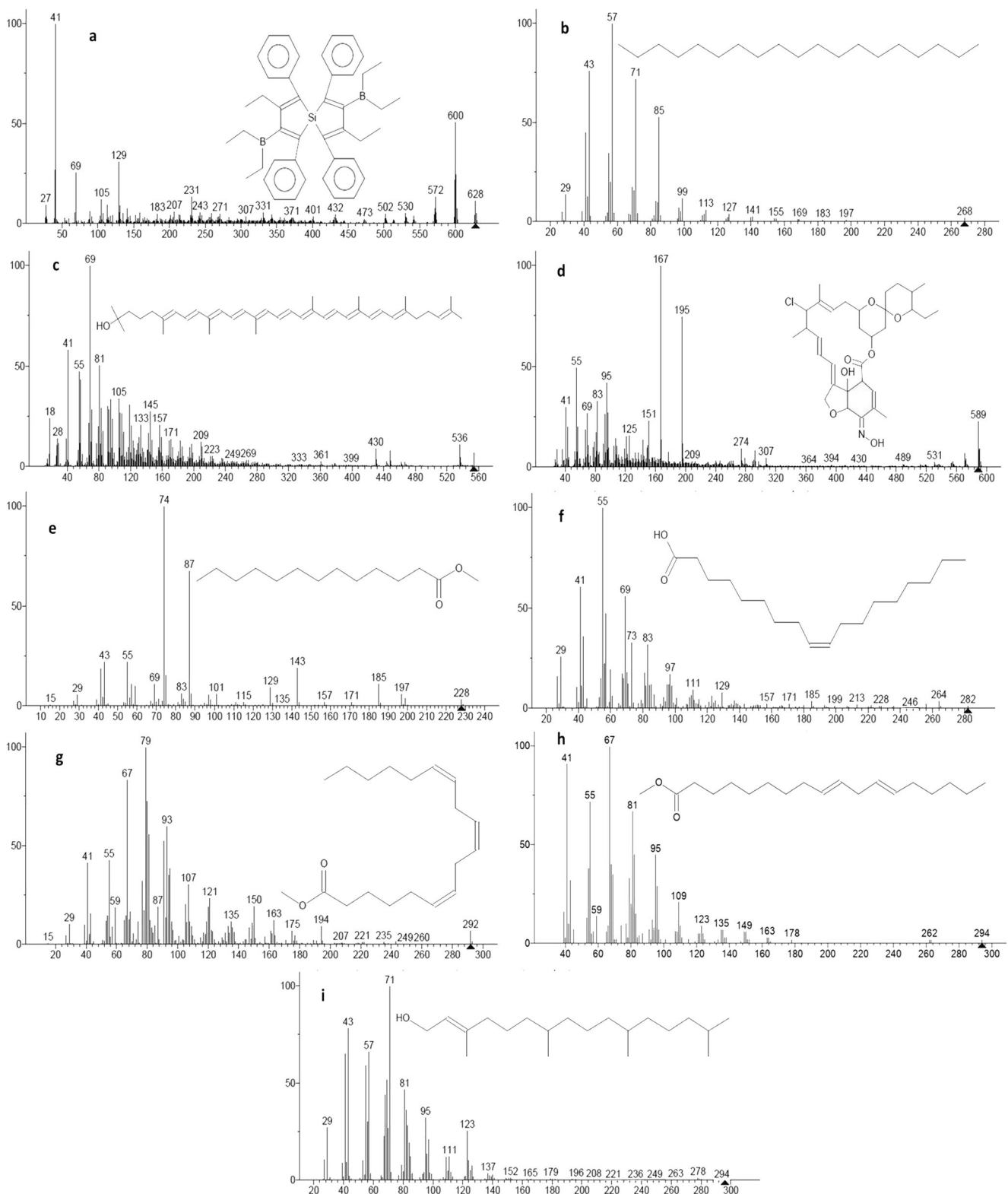
## Discussion

Algae are rich source of pigments, proteins and lipids, PUFA, polysaccharides, minerals, antioxidants, and many bioactive compounds (Ashour et al. 2019; Peixoto et al. 2019; Safavi et al. 2019; Sajina et al. 2019; Sharawy et al. 2020). The use of

algae and/or their extracts in aquatic animal feeds has resulted in improved growth performances and immunity enhancement (Sharawy et al. 2020).

In the current study, the proximate analyses of seaweed liquid extract TAM showed that it contains a large amount of macro-nutrients such as K (12%) and P (2.4%) and a small amount of total nitrogen (0.1%). Micro-nutrients like Cu, Fe, Mg, Zn, and Mn exist as traces. Moreover, heavy metals were absent in TAM (Table 3). Our finding agreed with Hamed et al. (2018) who found that seaweed extracts contains nutrient, trace minerals, and many bioactive materials. In addition, seaweed extract can contain vitamins, ascorbic acid, phyto-hormones, and other bioactive compounds.

The biochemical analysis of TAM showed that it contains 15% total polysaccharides, 8.2% total organic matter, and 2.6% total dissolved solids. Polysaccharides have demonstrated positive responses on growth performance, feed utilization, serum biochemical composition, immune responses, and disease resistance for many different cultured aquaculture species (Doan et al. 2017; Akbary and Aminikhoie 2018; Yengkhom et al. 2018). GC-Mass analysis showed that TAM seaweed liquid extract contains many valuable bioactive compounds (Table 4) not only for aquaculture feed additive but also possibly for many pharmaceutical applications. Methylated fatty acids (tridecanoic acid methyl ester; 9,12-octadecadienoic acid methyl ester, (E,E)-; and  $\gamma$ -linolenic acid methyl ester) and natural fatty acid (oleic acid) together comprised about 83% of the total peak area of TAM. Oleic acid is one of the most abundant fatty acids found in seaweeds (Matanjan et al. 2009; Cardoso et al. 2017). Diets rich in oleic acid have shown beneficial effects in inflammatory-related diseases and enhancing insulin production (Carrillo Pérez et al. 2012). Fatty



**Fig. 2** Mass spectra of nine phytochemical compounds identified in crude TAM. **a** 5-Silaspiro[4.4]nona-1,3,6,8-tetraene, **b** bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl- (RT, 8.99 min); **b** nonadecane (RT, 16.31 min); **c** rhodopin (RT, 19.45 min); **d**

milbemycin-oxime (RT, 20.07 min); **e** tridecanoic acid methyl ester (RT, 20.90 min); **f** oleic acid (RT: 21.63 min); **g**  $\gamma$ -linolenic acid methyl ester (RT, 23.74 min); **h** 9,12-octadecadienoic acid methyl ester, (E,E)- (RT, 24.02 min), and **i** phytol (RT, 24.37 min)



**Table 5** Biochemical composition of *O. niloticus* at the end of feeding experiment

Diets	CP	EE	Ash
D <sub>0</sub> (control)	705 ± 0.15c	132 ± 0.12b	162 ± 0.03a
D <sub>0.5</sub>	708 ± 0.29c	133 ± 0.09b	157 ± 0.28a
D <sub>1</sub>	721 ± 0.15b	137 ± 0.31ab	142 ± 0.40b
D <sub>1.5</sub>	720 ± 0.18b	138 ± 0.23ab	139 ± 0.38b
D <sub>2</sub>	729 ± 0.21a	140 ± 0.23a	130 ± 0.32c

Data were represented as means ± SD. Different lowercase letters in each column indicate significant differences (*P* < 0.05)

D<sub>0</sub>, diet supplemented with 0% TAM; D<sub>0.5</sub>, diet supplemented with 0.5% TAM; D<sub>1</sub>, diet supplemented with 1% TAM; D<sub>1.5</sub>, diet supplemented with 1.5% TAM; D<sub>2</sub>, diet supplemented with 2% TAM; CP, crude protein; EE, ether extract (% of dry matter basis)

acid methyl esters (FAMES) also have shown antioxidant, antibacterial, and antifungal activities (Agoramoorthy et al. 2007; Chandrasekaran et al. 2008; Pinto et al. 2017). Phytol is a product of chlorophyll metabolism in seaweeds and has demonstrated strong antioxidant and antinociceptive effects (Santos et al. 2013). Rhodopin is a carotenoid compound existing in marine seaweeds and has an antioxidant activity (Hirayama et al. 1994; Mohy El-Din and El-Ahwany 2016). Nonadecane is a natural alkane hydrocarbon reported in many red, brown, and green seaweeds harvested from the Alexandria coast showing antioxidant, anticancer, antifungal, antibacterial, and anti-inflammatory properties (Ibrahim et al. 2016; Shobier et al. 2016; Abdel-Latif et al. 2018; Hassan and Shobier 2018; Mohy El-Din and Mohyeldin 2018). The milbemycin groups (macrocyclic lactones) are reported to have highly potent antiparasitic, anthelmintic, and insecticidal activities (Prichard et al. 2012; Kumar et al. 2015). Milbemycin has also been used in treating infected aquatic animals to control and resist parasitic nematode and fish lice (Killino and Bodri 1997; Tang et al. 2019). Milbemycin oxime is a natural antibiotic originally from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* (Takiguchi et al. 1980; Okazaki 2006); however, milbemycins have not been previously detected in seaweeds. Therefore, more

investigations are needed to elucidate if the seaweed extracts like TAM are able to produce milbemycins and could become an alternative source of antiparasitic compounds commonly used in veterinary medicine. Another finding, 5-silaspiro[4.4]nona-1,3,6,8-tetraene,3,8-bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl, a phytochemical bioactive compound was detected in TAM. This compound (C<sub>44</sub>H<sub>50</sub>B<sub>2</sub>Si, exact mass weight 628.38) has two atoms of boron attached with one atom of silicon. Boron is an essential micronutrient required for embryonic development and bone metabolism of fish and recently reported as the immune response enhancer in *O. niloticus* (Ardo et al. 2008; Prabhu et al. 2016; Oz et al. 2018). Like milbemycin, there is no previous study showing the detection of this compound in seaweeds.

In the current study, growth performance, feed utilization, and carcass composition of *O. niloticus* increased significantly (*P* ≤ 0.05) with increasing TAM levels in the diet. This positive effect may be due to the bioactive phytochemical compounds detected in this seaweed extract like polysaccharides which have antioxidant properties. Antioxidants play an important role to protect shrimp and fish tissues from oxidative damage and are usually affected by feed composition (Sharawy et al. 2020). Additionally, the extraction process used enhances the ingestion, feed intake, and food conversion ratios by releasing bioactive compounds from inside the algal cell and making them available and thus enhancing the growth performance, blood parameters, and immunity of the fish. Many studies reported that the high contents of triacylglycerol, glycosides, PUFA, essential oil, vitamins, terpenoids, alkaloids, hydrocarbon, and sterols have positive effects on growth performance, feed utilization, carcass composition, blood parameters, immunity response, and antioxidant and antibacterial properties (Ashour et al. 2018; Sharawy et al. 2020), and many studies have shown that diet supplemented with seaweed enhances growth performance, feed utilization, carcass composition, survival rate, blood indices, and disease resistance in fish (Guroy et al. 2007; Ergun et al. 2009; Wassef et al. 2013; Khalafalla and El-Hais 2015). These effects could be attributed to several factors as follows: (i) seaweeds contain antimicrobial

**Table 6** Physiological parameters of *O. niloticus* fed on supplemented diets with different levels of TAM

Treatment	Total serum protein (g dL <sup>-1</sup> )	Albumin (g dL <sup>-1</sup> )	Globulin (g dL <sup>-1</sup> )	Triglycerides (g dL <sup>-1</sup> )	Glucose (g L <sup>-1</sup> )	GPT (U L <sup>-1</sup> )
D <sub>0</sub> (control)	4.53 ± 0.120c	1.86 ± 0.030b	2.67 ± 0.15c	200.33 ± 2.4c	108.33 ± 0.60a	43.33 ± 2.33a
D <sub>0.5</sub>	4.74 ± 0.050ab	1.94 ± 0.012b	2.80 ± 0.05bc	195.00 ± 3.1ab	107.67 ± 0.88a	44.33 ± 2.91a
D <sub>1</sub>	4.81 ± 0.060b	1.95 ± 0.015b	2.86 ± 0.07bc	190.33 ± 1.2bc	104.67 ± 0.86a	46.3 ± 1.76a
D <sub>1.5</sub>	5.24 ± 0.015a	2.11 ± 0.020a	3.13 ± 0.02ab	187.33 ± 0.8c	104.33 ± 1.20a	45.33 ± 1.45a
D <sub>2</sub>	5.48 ± 0.120a	2.097 ± 0.04a	3.38 ± 0.16a	179.67 ± 1.8d	99.33 ± 0.88a	43.67 ± 1.20a

Data were represented as means ± SD. Different superscript letters in each column indicate significant differences (*P* < 0.05)

D<sub>0</sub>, diet supplemented with 0% TAM; D<sub>0.5</sub>, diet supplemented with 0.5% TAM; D<sub>1</sub>, diet supplemented with 1% TAM; D<sub>1.5</sub>, diet supplemented with 1.5% TAM; D<sub>2</sub>, diet supplemented with 2% TAM

**Table 7** Serum lysozyme activity, respiratory burst activity, and the cumulative mortality of *O. niloticus* fed on supplemented diets with different levels of TAM

Diets	Lysozyme activity ( $\mu\text{g mL}^{-1}$ )	Respiratory burst activity ( $\text{mg mL}^{-1}$ )	Cumulative mortality (%)	RPS %
D <sub>0</sub> (control)	1.46 ± 0.03e	0.93 ± 0.00e	83.3	0
D <sub>0.5</sub>	2.77 ± 0.02d	1.48 ± 0.10d	26.7	68
D <sub>1</sub>	3.38 ± 0.04c	1.63 ± 0.30c	16.7	80
D <sub>1.5</sub>	3.51 ± 0.02b	1.82 ± 0.10b	13.3	85
D <sub>2</sub>	3.75 ± 0.03a	1.89 ± 0.20a	10	88

Data were represented as means ± SD. Different superscript letters in each column indicate significant differences ( $P \leq 0.05$ )

D<sub>0</sub>, diet supplemented with 0% TAM; D<sub>0.5</sub>, diet supplemented with 0.5% TAM; D<sub>1</sub>, diet supplemented with 1% TAM; D<sub>1.5</sub>, diet supplemented with 1.5% TAM; D<sub>2</sub>, diet supplemented with 2% TAM; RPS, relative percent survival

components (El-Shenody et al. 2019); (ii) seaweeds have an efficient antioxidant capacity (Leonard et al. 2011), (iii) seaweeds increase the antioxidant status by lowering lipid peroxidation, increasing glutathione peroxidase activity, and reducing stress indicators (Kannan et al. 2007); and (v) presence of bioactive compounds in the methanolic extract, such as phytohormones and polysaccharides which have an immune-stimulatory effects and disease resistance (Akbarly et al. 2018).

Total serum protein, albumin, globulin, glucose, and triglyceride levels are considered key factors in enhancing the health of immune system and blood functions, also considered useful tools for assessing the nutritional status and are well known for retaining strong innate immune system (Madibana et al. 2017; Akbarly et al. 2018). The current study reported that the additions of different levels of TAM significantly ( $P \leq 0.05$ ) enhanced the total serum protein, albumin, and globulin (Table 6). Our results are in agreement with Akbarly et al. (2018) who found that the addition of different levels of methanolic extract of *Ulva rigida*, as a dietary supplement enhanced total serum protein, albumin, and globulin and biochemical response of *Mugil cephalus*. The enhancement of plasma total protein level and the non-specific immune response of Nile tilapia may be due to the boron-containing compounds. For example, Ardo et al. (2008) observed that diet supplemented with boron-enhanced growth, survival, plasma total protein level, and the non-specific immune response of Nile tilapia and disease resistance against *A. hydrophila* (Prabhu et al. 2016; Oz et al. 2018).

The present study also found that triglycerides were significantly decreased with increasing of TAM levels (Table 6), as also observed by Akbarly et al. (2018). In addition, no significant differences were observed in glucose and/or GPT concentrations (Table 6).

The present study showed that serum lysozyme activity and respiratory burst assay were significantly ( $P \leq 0.05$ ) increased with increasing levels of TAM (Table 7). As well as, the survival of Nile tilapia *O. niloticus* challenged with *A. hydrophila* was significantly ( $P \leq 0.05$ ) increased with the

increase of TAM levels (Table 7). These results may be due to the phytochemical compounds which have antioxidant activities. Our findings are similar to those of Castro et al. (2004). In addition, Yengkhom et al. (2016) concluded that methanol extract of *Caulerpa scalpelliformis* injected intraperitoneally at four levels (2 to 200 mg kg<sup>-1</sup> body weight) in *O. niloticus* increased serum lysozyme, increased lysozyme gene expression and myeloperoxidase and antiprotease activities. Furthermore, Quezada-Rodriguez and Fajer-Avila (2017) reported that dietary supplementation of the polysaccharide ulvan extracted from *Ulva clathrata* contributes to an increase in the immune response of *O. niloticus*.

## Conclusion

The current study concluded that the use of the commercial seaweed liquid extract TAM, based on *U. lactuca*, *P. capillacea*, and *J. rubens*, as a feed dietary supplement of Nile Tilapia (*O. niloticus*) fingerlings significantly enhanced growth performance, feed utilization, and immune responses of *O. niloticus* challenged with *A. hydrophila*. The seaweed liquid extract (TAM) is a rich source of several bioactive phytochemical compounds which are suggested to be responsible for effects on fish. Finally, seaweed extracts are suggested as preferable feed additive supplements rather than whole cells to capitalize on the benefits of the bioactive compounds in the algal cells.

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