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The Potential of a New Commercial Seaweed Extract in Stimulating Morpho-Agronomic and Bioactive Properties of *Eruca vesicaria* (L.) Cav.

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Abstract: This study aimed to understand the effect of commercial seaweed extract as a biofertilizer, named True-Algae-Max (TAM®), on the yield, nutritional, antioxidant, and cytotoxic activity of *Eruca vesicaria*. Three concentrations of TAM® (5, 10, and 15%) were studied by foliar spray over the two cultivation years (2016 and 2017) without any chemical fertilizer, along with a control consisting of synthetic nitrogen, phosphorus and potassium (NPK) fertilizers. The yield and composition of *E. vesicaria* were significantly improved in all treatments, particularly at 10% concentration of TAM®, which resulted in maximum yield (1.99 kg m^{-2}) and significant amounts of chlorophyll, carotenoids, phenolic compounds, flavonoids and total nutrients. Compared to the NPK control, *E. vesicaria* grown with 10% of TAM® improved total antioxidant activity from 41.80 to 49.36 mg g^{-1} and cytotoxicity from 25.30 to 60.40% with an IC_{50} value 85.7 µg mL^{-1} against the hepatocellular carcinoma cell line (HepG2). These findings indicate that seaweed extract can generally be used as a safe potential multifunctional biofertilizer in the agricultural field. The use of seaweed as a biofertilizer could potentially help mitigate the adverse effects of main nutrient deficiencies, diminishing the use of chemical fertilizers.

Keywords: antioxidant; biofertilizer; growth regulators; cytotoxicity; HepG2; *Eruca vesicaria*; seaweed extract; TAM®; 5-Silaspiro[4.4]nona-; phytol; rhodopin; nonadecane

1. Introduction

With rapid growth in the global population and increasing demand for foods with good nutritional and health values, there is growing pressure to produce sufficient food [1,2]. On the other hand, essential nutrients required for crop growth are gradually depleting from the soils, which results in lower crop yields per unit area of land [3]. As attempts to overcome these issues, chemical fertilizers are widely used. Although chemical fertilizers can increase the crops' growth and yields, their overuse has various side effects, including hardening the soil, decreasing soil fertility, strengthening pesticides, and polluting water [4]. Moreover, these chemicals tend to increase the susceptibility of plants to pathogens by altering the soil microbiome composition and substantially influencing plant health and

eventually pose a serious threat to consumers [5] as well as the whole ecosystem in the long run [6]. Therefore, modern agricultural practice is seeking alternatives to chemical fertilizers.

A promising approach is the use of biofertilizers in the form of mixtures of natural compounds, which could stimulate the growth and yield of plants and improve the tolerance efficiency to abiotic and biotic stresses with no side effect [7–9]. Marine organisms are good sources for bioactive compounds which have wide ranges of biological activities [10–14]. In this context, macroalgae would be potential options as biofertilizers [15,16]. In general, macroalgae are treasure chests of many organic and inorganic components, improving plants' quantity and quality by enhancing plant growth, protection and immune stimulation [8,13,17]. In this aspect, screening native aquatic organisms should be considered to achieve a thriving commercial and biotechnological potential [18]. Algal cells are considered an attractive aquatic natural source of bioactive materials that can be successfully utilized in several applications [19–21]. However, Egypt has a wide variety of wild seaweeds throughout the year, either along the Mediterranean coast [22–26] or the Red Sea coast [27,28]. Along the Egyptian Mediterranean coast, especially the Alexandria coast, green algae such as *Ulva lactuca* (Chlorophyta) and red algae such as *Jania rubens* and *Pterocladiella capillacea* (Rhodophyta) are the most dominant native seaweed species.

Eruca vesicaria (L.) Cav. is becoming a significantly important leafy salad crop across the world. *E. vesicaria* (L.) Cav. (formerly *E. sativa* Mill.) belong to the Brassicaceae family (*Cruciferae*) and are widely used as a source of healthy phytochemicals and nutraceutics [29]. *E. vesicaria* seems to be a promising industrial food crop as it has many bioactive phytochemicals [30–32], making it a promising source for antioxidants and cytotoxicity properties for health and cosmetics applications [33–38]. Despite the fact that these seaweed extracts and their physiological effects have been widely described, the impact of these extracts on the bioactive components and enhancing their activities have been poorly studied and still need more investigation.

Therefore, the present study aimed to evaluate the suitability of the mixed seaweed extract as a commercial biofertilizer for Rocket salad, *E. vesicaria* (L.) Cav. In this context, a comparative evaluation was made on the performances between seaweed biofertilizers (three treatments without chemical NPK application) and chemical fertilizer (NPK control), considering the vegetative growth, yield, nutrient contents, and bioactive compounds. Further experiments were conducted to study the effects of the seaweed extract to improve antioxidant and cytotoxic activities of the *E. vesicaria* (L.) Cav.

2. Materials and Methods

2.1. Materials

2.1.1. Algae Source

A commercial seaweed liquid biofertilizer, namely True-Algae-Max (TAM®), that was prepared and used in the current study has been submitted at the Academy of Scientific Research and Technology (Egypt Patents Office, Cairo, Egypt submission No.: 2046/2019) [39]. TAM® was prepared from the extract of the green alga *Ulva lactuca* (Linnaeus) and red algae *Jania rubens* (Linnaeus) and *Pterocladiella capillacea* (S.G. Gmelin) Santelices. These seaweeds were collected from Boughaz El-Maadya, Abu-Qir Bay (31°16′16.0″ N, 30°10′28.0″ E), Alexandria, Egypt. These three species are the most dominant native species along the Egyptian Mediterranean coast of Alexandria [23,24,40].

2.1.2. Plant Material

E. vesicaria seeds of local Egyptian cv. Balady were purchased from the Egyptian Ministry of Agriculture stores and stored at 4 °C in a plastic bag containing silica gel until sown.

2.2. Methods

2.2.1. Phytochemical Analysis of TAM®

The crude TAM® was tested for physicochemical properties (color, odor, density, pH, organic matter, polysaccharides and total dissolved solids), macronutrients (N, P, K and Mg), micronutrients (Cu, Fe, Zn and Mn), and heavy metals (Cd, Cr, Pb, Ni and Ar) according to the standard methods as the following—total macro, micronutrients and heavy metals were determined in the seaweed extracts by using an Inductively Coupled Plasma Spectrometer (Perkin Elemer Emission Specrrophotometer- 6000 Series, Thermo Scientific) [41]. Total polysaccharides were determined using the micro phenol-sulfuric acid method [42], with glucose as a standard. Total organic matter was determined according to the method of Albrektienė [43]. GC-Mass Spectrophotometry analysis was performed according to Elshobary et al. [44]. NIST library was used to identify the unknown compounds. According to Ashour et al. [45], physical properties, chemical and biochemical composition, and phytochemical compounds of crude TAM® are presented in Tables 1 and 2.

Table 1. Physical, chemical and biochemical analyses of True-Algae-Max (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	
Macroelements	
Potassium (%)	12
Phosphorus (%)	2.4
Total nitrogen (mg/kg)	1400
Microelements (mg/kg)	
Copper	0.39
Iron	16.18
Magnesium	19.72
Zinc	1.19
Manganese	3.72
Heavy metals (mg/kg)	
Cadmium	LOQ **
Chromium	LOQ **
Lead	LOQ **
Nickel	LOQ **
Arsenic	0.55

* Cited from Ashour et al. [45]. ** LOQ: Less than the limit of quantification.

Table 2. Phytochemical constituents of crude TAM®.*.

Peak No	Retention Time	Compound Name	Phytochemical Group	Formula	Molecular Weight	Content %	Applications	Ref.
1	9.022	5-Silaspiro [4.4]nona-1,3,6,8-tetraene,3,8-bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl	Silanes	$C_{44}H_{50}B_2Si$	628.38	1.93%	Immune response enhancer	[45,46]
2	16.824	Nonadecane	Alkane hydrocarbon	$C_{19}H_{40}$	628.39	3.61%	Antioxidant, antimicrobial activities	[45,47–49]
3	19.284	Rhodopin (6E,8E,10E,12E,14E,16E,18E,20E,22E,24E,26E)-2,6,10,14,19,23,27,31-octamethyldotriaconta-6,8,10,12,14,16,18,20,22,24,26,30-dodecaen-2-ol)	Carotene	$C_{40}H_{58}O$	268.31	0.81%	Antioxidant activity, immune response enhancer	[46,48,50]
4	20.071	Milbemycin A4 5-oxime (1R,4S,5′S,6R,6′R,8R,10E,13R,14E,16E,20R,24S)-6′-ethyl-24-hydroxy-21-hydroxyimino-5′,11,13,22-tetramethylspiro[3,7,19-trioxatetracyclo[15.6.1.14,8.020,24] pentacosa-10,14,16,22-tetraene-6,2′-oxane]-2-one)	Macrocyclic lactones	$C_{32}H_{44}ClNO_7$	589.28	4.75%	Antiparasitic and insecticidal activities	[45,51,52]
5	20.514	Octadecenoic acid methyl ester (9,12-octadecadienoic acid, methyl ester, (E,E))	Methylated fatty acids	$C_{17}H_{32}O_2$	554.45	52.20%	Antioxidant activities	[44–46]
6	20.901	Tridecanoic acid methyl ester		$C_{14}H_{28}O_2$	268.24	2.79%		
7	23.748	γ-Linolenic acid methyl ester (6,9,12-Octadecatrienoic acid, methyl ester)		$C_{19}H_{32}O_2$	228.21	14.78%		
8	21.627	Oleic Acid (cis-9-Octadecenoic acid)	Fatty acid	$C_{18}H_{34}O_2$	292.24	12.55%	Antioxidant activities	[44]
9	24.295	Phytol (3,7,11,15-Tetramethylhexadec-2-en-1-ol)	Phytol	$C_{20}H_{40}O$	294.26	6.59%	Antioxidant activities	[45,53]

* Cited from Ashour et al. [45].

2.2.2. Field Experiment and Soil Analysis

Field experiment with *E. vesicaria* (L.) Cav. was conducted for two successive years in 2016 and 2017 at the Abeis Experimental Farm Station, Faculty of Agriculture, Alexandria University, Egypt (31°11′25.9″ N 30°00′25.1″ E). The average maximum and minimum temperatures in the experimental period were 25 and 15 °C, respectively. Average relative humidity was 70 ± 5%, with an average monthly rainfall of 250 ± 5.5 mm, during cultivation season (March–May). *E. vesicaria* seeds were sown on the 25th March in both years. Before sowing the seeds, physical and chemical properties of the soils collected from up to 30 cm depth were determined by the standard procedures. To determine soil pH, 10 g of soil was mixed with 1:5 distilled water and the pH was determined using a pH meter. The total nitrogen content of 1 g of soil was determined using the micro-Kjeldahl process. The Lancaster method was used to calculate the amount of available phosphorus (P_2O_5) and nitrogen content in the soil. Potassium, calcium, magnesium, and sodium exchangeables were eluted with 1 N NH4OAc and then analyzed with a spectrophotometer. Regarding anions analysis, bicarbonate is generally determined in soil saturation extract by titration with 0.01 N H_2SO_4 to pH 4.5, respectively [54]. Soluble chloride is measured in the saturation extract of soil by silver nitrate titration [54]. Sulfate is determined by the wet digestion method with acid mixture (nitric: perchloric: sulfuric acid) at the ratio of (8:1:1) [54].

2.2.3. Treatments and Experimental Design

Three foliar spray concentrations (5, 10 and 15%) of TAM® were studied as the treatments without NPK application, while standard amounts of NPK chemical fertilizer were used as the control. The experimental layout was a randomized complete block design, as also studied earlier [55]. The experimental area was 150 m^2 with three replicates. The area of each plot was (5 × 2.5 m^2) 12.5 m^2. The seeds were sowed by the broadcasting method at an amount of 28 kg h^{-1} [16]. The plots were randomly arranged under the sunlight at 22 ± 4 °C at mid-day. TAM® concentrations were added as foliar spray three times after planting at the 10th, 18th, and 26th days 2000 mL per plot (100–200 mL/m^2) for

each cut. The total amount of N, P and K of crude TAM® was 3136, 5376 and 2688 kg ha^{-1}, respectively. NPK chemical fertilization was carried out according to the recommendations for commercial production of *E. vesicaria* plant [56]. The NPK treatment dose consisted of ammonium sulfate (20.5%N) at the rate of 529 kg ha^{-1}, calcium superphosphate (15% P_2O_5), 302 kg ha^{-1} and potassium sulfate (48% K_2O), 126 kg ha^{-1}. Nitrogen fertilizer was applied thrice on the 10th, 18th, and 26th days. Phosphorus fertilizer was mixed during soil preparation. Potassium fertilizer was applied for 15 days.

2.2.4. Determination of Growth and Yield Parameters

In both years, the plants were harvested (cut) twice, at 35 and 65 days of sowing, from each plot (treatment) to determine total yield (kg^{-2}). Five plants were randomly selected from each treatment of three replicates (15 samples) to measure plant height (cm), dry leaf weight (g), and the number of leaves per plant (No.).

2.2.5. Determination of Chlorophyll, Carotenoid, and Mineral Contents

Pigment contents in the plants were determined by extracting 0.2 g of fresh leaf sample in 25 mL of 80% acetone for 48 h at room temperature in the dark. Absorbances were read at 662, 645, and 652 nm in a spectrophotometer (UV-3802, UNICO, Dayton, Ohio, USA). Chlorophyll a, b and carotenoid were calculated using Equations (1)–(4) [57]:

$$Chlorophyll\ a = (11.75 \times A_{662}) - (2.69 \times A_{645}) \quad (1)$$

$$Chlorophyll\ b = (18.61 \times A_{645}) - (3.960 \times A_{662}) \quad (2)$$

$$Total\ chlorophyll = (Chl.\ a + Chl.\ b) \quad (3)$$

$$Total\ carotenoid\ content = (1000 \times A_{470} - 2.270 \times Chl.\ a - 81.4 \times Chl.\ b)/227 \quad (4)$$

where A is the absorbance of the sample on the spectrophotometer. The results were expressed as micrograms per gram fresh weight of the sample.

Nutrient contents (N, P, and K) in *E. vesicaria* were determined as a percentage of the dry weight of the leaves. Total nitrogen and phosphorus contents were determined using the spectrophotometric method [58], while potassium was determined in the atomic absorption spectrometry [59].

2.2.6. Preparation of Plant Extracts

The harvested samples of *E. vesicaria* in 2016 and 2017 were oven-dried at < 45 °C till constant weight (48 h) and then ground into fine powder. One gram of the plant powder (three replicates for each treatment) was extracted with 10 mL methanol for 10min in a sonication bath and then kept in methanol (90%) for 24 h at room temperature for further extraction [60]. These extracts were filtered with a Whatman® No. 1 filter paper. Filtrates were evaporated to dryness and resuspended in methanol to 100 μg mL^{-1} to evaluate the antioxidants and cytotoxic activities.

2.2.7. Determination of Antioxidant Activity and Phytochemicals

Free radical scavenging activity of the methanolic extract was performed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method described by Viturro et al. [61]. Total antioxidant activity (TAA) was determined by the phosphomolybdate method using ascorbic acid (μg mL^{-1}) as the standard [62]. Total phenolic content (TPC) was determined by the Folin–Ciocalteu method modified from Kumar et al. [63]. Total flavonoid content (TFC) was estimated by the method described earlier [64] using quercetin as the standard.

2.2.8. Determination of Cytotoxic Activity

Cytotoxic activity of the *E. vesicaria* crude methanolic extracts (after evaporating methanol) was obtained from TAM® (three treatments) and the control using lung cancer cell line (A549) and hepatocellular carcinoma cell line (HepG2). Cell cultures were

maintained in Dulbecco's modified Eagle's medium (DMEM) (ATCC 30-2002) for the A549 cell line and Roswell Park Memorial Institute medium (RPMI-1640) for the HepG2 cell line. The culture media were supplemented with 10% fetal bovine serum and incubated at 37 °C under 5% CO_2 and 95% humidity. After that, a sub-culture of the cell lines was achieved using 0.15% trypsin-versene. In 96 well plates, 10^4 cells (obtained after 24 h culturing) were taken separately in each well for both A549 and HepG2 cell lines containing 100 µL respective serum-free media of each cell line. The final concentration of the plant extracts in each respective well was 100 µg mL^{-1}. The experiments were performed in triplicate at 37 °C for 48 h. Doxorubicin and 0.5% dimethyl sulfoxide (DMSO) were used as the positive and negative controls, respectively. Cell viability was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [65] by adding 50 µL of MTT solution (5 mg mL^{-1}) into the treated cells to form formazan crystals within the living cell. To separate the formazan crystals, the mixtures were centrifuged at 8000 rpm for 10 min. The supernatant was disregarded, and 1 mL of DMSO was added to dissolve the precipitated formazan crystals. Absorbance was detected in a microplate reader (Model 680) at 595 nm**.** Cytotoxicity was calculated for all treatment and controls using Equation (5):

$$Cytotoxicity\ (\%) = (1 - A_X/A_{NC}) \times 100 \quad (5)$$

where A_X and A_{NC} are the absorbance of the sample and control at OD_{595}, respectively.

The 50% inhibitory concentration (IC_{50}) was determined using different concentrations of the highly active extracts showing high cytotoxicity on the cancer cell lines.

2.2.9. Statistical Analysis

A comparison among the factors was statistically made by one-way analysis of variance (ANOVA) with Duncan post hoc test, as well as two-way ANOVA using plant parameters as a dependent factor, while treatments, years and their interaction were used as independent factors using the IBM SPSS Statistics software (IBM, v.23). Differences among means were considered significant at $p < 0.05$. Pearson's correlation coefficient was determined for the relationship between phytochemical compounds and their biological activities in SPSS software. All morphometric and phytochemical parameters of untreated and TAM® treated plants were subjected to the principal component analysis (PCA) to discover relationships among parameters and treatments. We determined the effective treatment that gives the maximum yield and quality among TAM® concentrations using Paleontological Statistics (PAST3).

3. Results

3.1. Soil Analysis

Regarding the soil analysis, it was observed that the difference in physical properties between the two cultivation years was not significant at $p < 0.05$ using Duncan's multiple range test (Table 3).

3.2. Morpho-Agronomic Properties

The results showed that morpho-agronomic traits of *E. vesicaria* varied among the treatments and NPK control ($p < 0.05$), but variations in the parameters were not significant between the two experimental years ($p < 0.05$), except the plant height and number of leaves that varied significantly between the years at $p < 0.05$ using two-way ANOVA (Table A1). Plant height was increased with 10% and 15% TAM by 1.17 and 1.23 times over the control, while plant height with the NPK control was comparable to that with 5% seaweed extract (Table 4).

Table 3. Physical and chemical properties of soil of the experimental field determined before cultivation in 2016 and 2017.

Soil Parameters	2016	2017
Particle size distribution		
Sand (%)	32.3 ± 1.6	31.5 ± 1.2
Silt (%)	25.2 ± 2.5	28.7 ± 2.2
Clay (%)	42.5 ± 3.6	39.8 ± 3.1
Soil texture	Clay loam	Clay loam
pH	7.45 ± 0.5	7.35 ± 0.3
Chemical Properties		
Soluble Cations (mmol g^{-1} soil)		
Ca^{2+}	1.44 ± 0.4	1.40 ± 0.5
Mg^{2+}	1.45 ± 0.4	0.98 ± 0.2
Na^{+}	3.63 ± 0.5	4.75 ± 0.7
K^{+}	0.54 ± 0.05	0.36 ± 0.03
Soluble Anions (meq L^{-1})		
HCO^{3-}	1.66 ± 0.1	1.78 ± 0.2
Cl^{-}	2.00 ± 0.3	1.80 ± 0.2
$SO_4{}^{2-}$	1.70 ± 0.5	1.65 ± 0.6
Total nitrogen (TN) (%)	0.16 ± 0.03	0.15 ± 0.01
Available phosphorus (mg L^{-1})	0.32 ± 0.02	0.27 ± 0.01

Each value is the mean of three replicates ± SD. All data showed no significant differences ($p < 0.05$) using Duncan's multiple range test.

Table 4. Plant structure and yield of *E. vesicaria* treated with different foliar spray concentrations of TAM®, compared with NPK control treatment.

Parameters	Cultivated Year	TAM® Treatments			
		0% (Control)	5%	10%	15%
Plant height (cm)	2016	34.00 ± 2.65 ^{b}	33.67 ± 2.31 ^{b}	40.00 ± 2.65 ^{a}	42.66 ± 0.58 ^{a}
	2017	39.60 ± 1.53 ^{BC}	37.00 ± 2.65 ^{C}	43.33 ± 1.13 ^{A}	42.67 ± 1.15 ^{AB}
Number of leaves (No.)	2016	7.00 ± 0.04 ^{c}	8.33 ± 0.60 ^{a}	7.66 ± 0.58 ^{b}	8.02 ± 0.03 ^{ab}
	2017	7.60 ± 0.55 ^{C}	8.00 ± 0.02 ^{B}	8.70 ± 0.58 ^{AB}	9.06 ± 1.00 ^{A}
Dry matter (%)	2016	17.12 ± 0.31 ^{a}	16.73 ± 0.65 ^{a}	15.07 ± 1.10 ^{b}	15.93 ± 0.39 ^{ab}
	2017	17.31 ± 1.48 ^{A}	16.75 ± 0.75 ^{AB}	15.38 ± 0.23 ^{B}	15.82 ± 0.43 ^{AB}
Total yield (kg m^{-2})	2016	1.61 ± 0.19 ^{b}	1.89 ± 0.02 ^{ab}	1.99 ± 0.47 ^{a}	1.82 ± 0.07 ^{ab}
	2017	1.59 ± 0.11 ^{B}	1.64 ± 0.27 ^{B}	2.28 ± 0.17 ^{A}	1.85 ± 0.13 ^{B}

Each value is the mean of three replicates ± SD. Different superscript letters in each row indicate significant differences ($p < 0.05$) using Duncan's multiple range test.

The different TAM® foliar extracts increased the number of leaves slightly over the NPK control treatment, which recorded the lowest number of leaves (seven leaves), where the maximum leaf number was observed with 15%, 5% and 10% TAM®, respectively (Table 4). Although the lowest dry matter was recorded with 10% seaweed extract, the results were comparable in all treatments, while with the NPK control, and in 2016 also for TAM® 5%, the results were significantly higher (Table 4). In 2016, the total yield of *E. vesicaria* was enhanced by the foliar TAM® spray over the NPK control. The highest increasing percentage (24%) was recorded with 10% TAM® over the NPK control. In 2017, the yield was higher than what was found in 2016, where the total yield was increased by 43% with 10% TAM® over the NPK control. The lowest yield was found in the NPK control in both years compared to any treatment (Table 4).

3.3. Pigment Content

It was observed that Chlorophyll a in *E. vesicaria* did not vary significantly among the treatments and NPK control as well as in both years ($p < 0.05$) (Figure 1). TAM® treatment did not affect the Chlorophyll b content compared with the control, and the 15% showed the lowest concentration and the highest content was observed with 5 and 10% TAM®. The

highest carotene content was found with 10% TAM® in both years, which was considered the best TAM® concentration to act as a biofertilizer**.** Indeed, it was observed that there was no significant difference in all pigment contents in both studied years (Table A1).

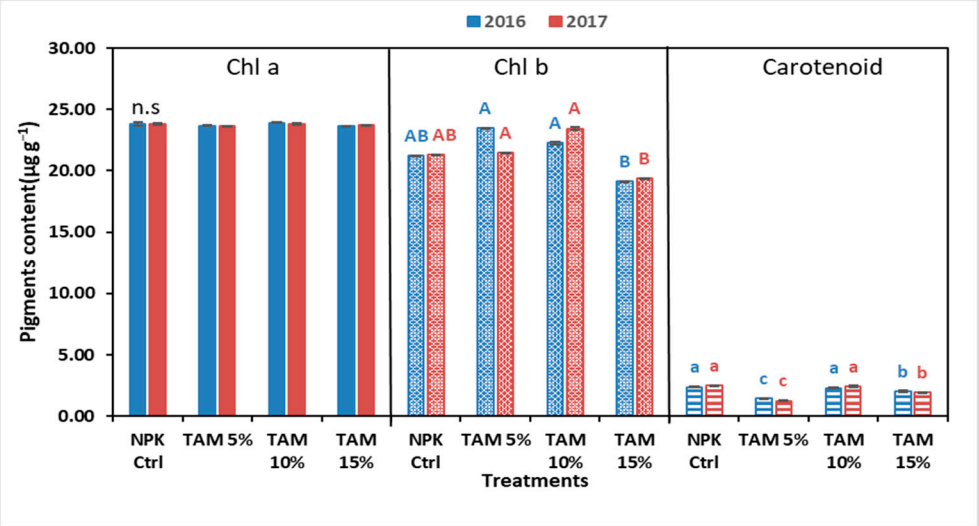

Figure 1. Pigment content in *E. vesicaria* treated with different foliar sprays of TAM® (5, 10, and 15%), compared with NPK control (TAM® 0%). Different letters in each plotted series indicate significant differences at $p < 0.05$ using Duncan's multiple range test. n.s., nonsignificant.

3.4. Nutrient Content

In general, there was no significant variation in nitrogen content in the plants between two years (Table A1). Treatments consisting of 10% TAM® provided the highest nitrogen accumulation in both years, while 15% TAM® and the NPK control showed the lowest value with no significant difference at $p < 0.05$ (Figure 2). Regarding the phosphorus content, all TAM® treatments showed phosphorus content close to that of the NPK control (Figure 2). However, potassium content increased significantly in the TAM® treatments, mainly with 15% TAM® (2.38% in 2016 and 2.32% in 2017), over the NPK control in both years. The differences were not statistically significant among treatments.

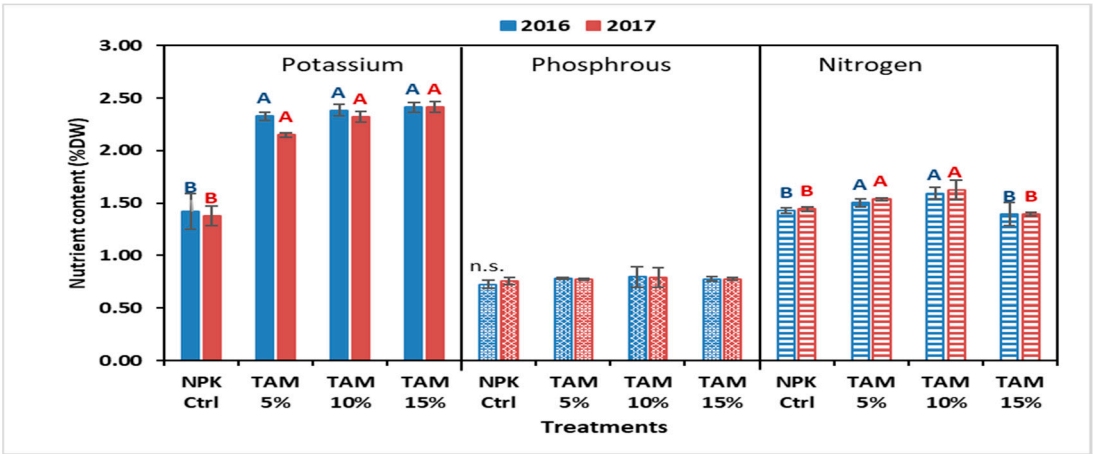

Figure 2. Nutrient content (%) in *E. vesicaria* treated with different foliar spray concentrations of TAM® (5, 10, and 15%), compared with NPK control (TAM 0%). Different letters in each plotted series indicate significant differences at $p < 0.05$ using Duncan's multiple range test. n.s., nonsignificant.

3.5. Phytochemical Content

As shown in Figure 3, maximum phenolic content in *E. vesicaria* was achieved with 10% TAM® (106.38 mg g^{-1} in 2016 and 105.52 mg g^{-1} in 2017), while there was no significant difference between 5% and 15% TAM® treatments. On the other hand, the lowest phenolic content was provided by the NPK control. Likewise, treatment with 10% TAM® showed the highest total flavonoids (2.94 mg g^{-1} in 2016 and 2.97 mg g^{-1} in 2017), while the control, 5% and 15% TAM® were comparable. It was observed that there was no significant variation in phytochemical content in both studied years (Table A1).

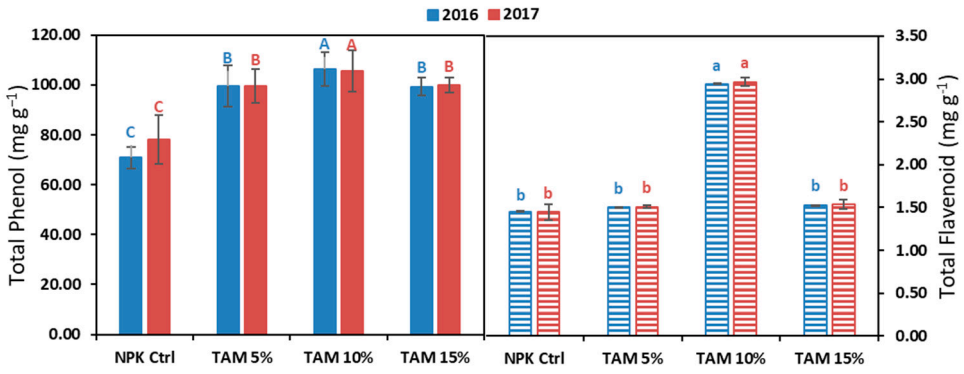

Figure 3. Phytochemical content in *E. vesicaria* treated with different foliar spray concentrations of TAM®, compared with NPK control treatment. Different letters in each plotted series indicate significant differences at $p < 0.05$ using Duncan's multiple range test.

3.6. Antioxidant Activity

The highest DPPH inhibition was achieved by 10% TAM® (63.63% in 2016), which was a little higher than the NPK control. However, 5% and 15% TAM® showed a lower DPPH scavenging activity than the NPK control in 2016, while in 2017, 5% TAM® showed comparable results with NPK treatment. Compared to the activity shown by the NPK control treatment, 10% TAM® showed the maximum total antioxidant activity (Figure 4). The highest total antioxidant activity was achieved with 10% TAM® (48.59 mg g^{-1} in 2016 and 52.43 mg g^{-1} in 2017), while the lowest activity was achieved with the NPK control. The same trend of antioxidant activities was observed in both years with no significance (Table A1).

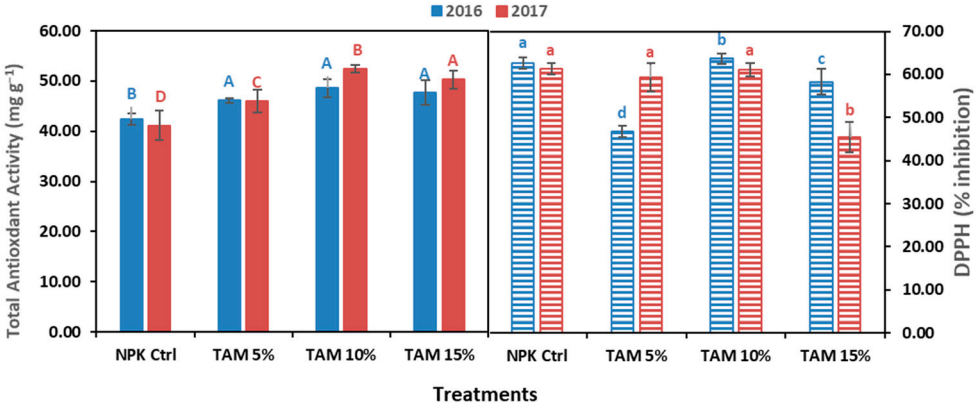

Figure 4. Antioxidant activity of *E. vesicaria* treated with different foliar spray concentrations of TAM®, compared with NPK control treatment. Different letters in each plotted series indicate significant differences at $p < 0.05$ using Duncan's multiple range test.

3.7. Cytotoxic Activity

Cytotoxic effects of methanolic extracts (dissolved in 0.05% DMSO) of *E. vesicaria* obtained from the different treatments of TAM® and the NPK control were estimated against the human cancer cells HepG2 and A549**.** Cultures of two cell lines were firstly treated with a methanol extract of *E. vesicaria* (100 µg mL^{-1}). Treatment with 10% TAM® showed 60.40% cytotoxicity against HepG2, which was more than 2-fold higher than that exhibited by the NPK control (Table 5). Likewise, TAM® 10% showed cytotoxic effect against the A549 cell line almost the same as the NPK control did. The IC_{50} value with 10% TAM® against HepG2 was found to be 85.7 µg mL^{-1} (Table 5). Noteworthily, the negative control showed no cytotoxic activity. The correlation coefficients (r) between the carotenoids with total phenolic and antioxidant activities were relatively high (0.91 and 0.96, respectively) (Table 6). On the other hand, cytotoxicity activity (HepG2) significantly correlated with carotenoids, total flavonoids and total antioxidant activity (0.73, 0.59 and 0.77, respectively) (Table 6).

Table 5. Cytotoxicity of methanol extracts (100 µg mL^{-1}) on human tumor cell lines, lung carcinoma (A549) and hepatocellular carcinoma (HepG2).

TAM Treatments	Cytotoxicity%	
	A549	HepG2
0% (Control)	27.33 ± 1.60 ^{b}	25.30 ± 0.72 ^{c}
5%	15.80 ± 1.39 ^{c}	13.20 ± 1.80 ^{d}
10%	27.00 ± 0.89 ^{b}	60.40 ± 1.61 ^{b}
15%	13.00 ± 1.00 ^{d}	12.10 ± 1.80 ^{d}
Doxorubicin	87.33 ± 0.61 ^{a}	88.83 ± 0.90 ^{a}
IC_{50}	ND	85.7 µg mL^{-1}

Represented data are mean ± SD. Different superscript letters in each column indicate significant differences ($p < 0.05$) using Duncan's multiple range test. ND, not detected.

Table 6. The Pearson correlation coefficient between the studied features of *E. vesicaria* extracts and its biological activities.

	TC	TPC	TFC	TAA	Cyto% (A549)	Cyto% (HepG2)
TC	1.00					
TPC	0.91 **	1.00				
TFC	0.44	0.38	1.00			
TAA	0.96 **	0.858 **	0.38	1.00		
Cyto% (HepG2)	0.73 **	0.44	0.59 *	0.77 **	1.00	
Cyto% (A549)	0.03	0.43	−0.04	−0.07	−0.58	1.00

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). TC: Total carotenoids; TPC: Total phenolic content; TFC: Total flavonoid content; TAA: Total antioxidant activity; Cyto% (A549) and Cyto% (HepG2): Cytotoxicity% (HepG2) and cytotoxicity% (A549), respectively.

3.8. Principal Components Analysis (PCA)

Three factors (PCs) were associated with Eigenvalues higher than one and accounted for 100% of the total variance. Factor 1 (PC1) explained 50.16% of the difference. The loading of the parameters on PC1 shows that plant height, number of leaves, total yield, phosphorous, potassium, total phenols, flavonoids and dry matter were the dominant variables, while plant height, Chl. a, carotenoids, Chl. b and nitrogen content were the dominant variables on the PC2, which explained 30.23%. PC3 explained the remaining 19.61% and showed Chl. a, Chl. b, and nitrogen as dominant variables (Table 7).

Table 7. Eigenvalues, variance percentages and loadings values of some variables on the axes identified by PCA for NPK control and TAM treatments of *E. vesicaria*.

Principal Components	PC 1	PC 2	PC 3
% variance	50.16	30.23	19.61
Eigenvalue	6.02	3.62	2.35
	Eigenvectors		
Plant height	0.63 *	0.65 *	−0.41
Leaves no.	0.85 *	−0.24	−0.48
Total yield	0.92 *	0.27	0.28
Dry matter	−0.93 *	−0.38	0.00
Chl. a	−0.02	0.72 *	0.70 *
Chl. b	−0.01	−0.65 *	0.76 *
Carotenoids	−0.17	0.97 *	0.19
Nitrogen%	0.26	−0.74 *	0.62 *
Phosphorus%	0.98 *	−0.13	0.13
Potassium%	0.83 *	−0.51	−0.23
Total phenols	0.98 *	−0.21	−0.04
Total flavonoids	0.70 *	0.44	0.57

The values with asterisks indicate the most significant characters for each principal component.

As shown in Figure 5, plotting data between PC1 and PC2 grouped 15% and 10% TAM® in the first upper positive quarter with plant height, total flavonoids and total yield, the NPK control in the second negative quarter with carotenoids and Chl. a, and 5% TAM® was located in the positive third quarter with mineral contents, the number of leaves and total phenols. In particular, regarding the interpretation of the components, it was observed that PC1 is strongly correlated with the morphometric trait variables including total yield, number of leaves and plant height, as well as nutrient and bioactive compounds such as P, K, total phenols and flavonoids. On the other hand, PC2 strongly correlated with carotene and Chl. a. However, PC3 was positively correlated with Chl. a, Chl. b and N% (Table 7).

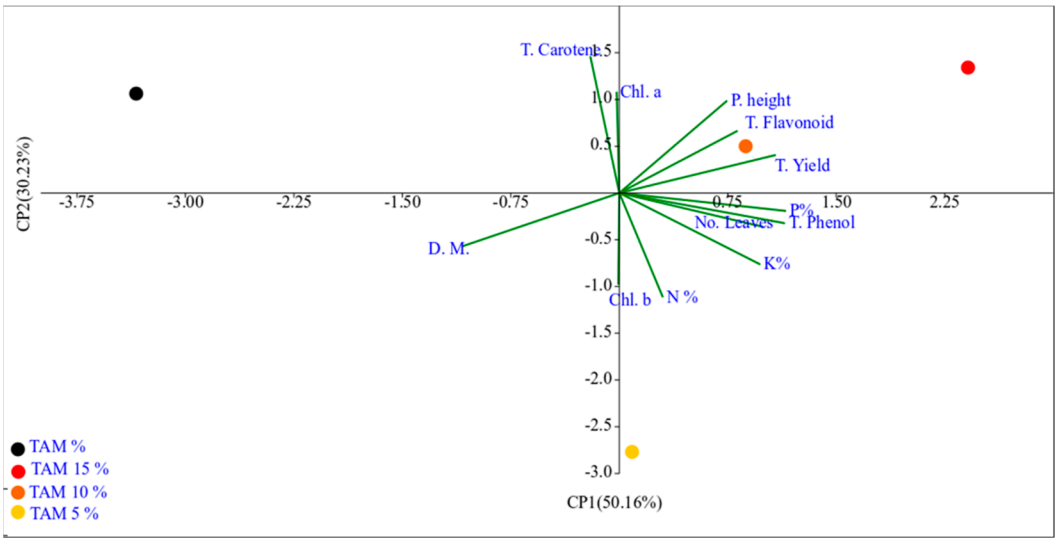

Figure 5. Principal component plot and scores of principal component analysis (PCA) for morpho-agronomic and biochemical traits of *E. vesicaria* in response to different foliar spray concentrations of TAM® (5, 10, and 15%), compared with NPK control (TAM® 0%). D.M.: dry mater, N: nitrogen, K: potassium, P: phosphorus, T: total, P: plant.

4. Discussion

Seaweed extracts have been shown much interest over the past few years as biofertilizers of various plants for improving plant growth and the yield of crops [66]**.** These extracts can be added directly to the soil or with irrigation water, while foliar spraying is a popular and widely used method of applying seaweed extracts [67,68]. The influences of these seaweed extracts on crops are mainly based on the availability, solubility and biological activity of the algal biomolecules and their extraction methods.

Physical, chemical, and biochemical properties of TAM® were evaluated before applying it as a biofertilizer in the cultivation of *E. vesicaria*, as described by Ashour et al. [45]. Physical investigations (Table 1) showed an appropriate amount of nutrients, macronutrients, and micronutrients. These findings indicated that these seaweed extracts had potential for use as organic fertilizers. In fact, the positive effects of any seaweed extract application as biofertilizers are the result of their chemical and biochemical constituents that work synergistically, although the mode of action is still unknown [45,47,67,69]. The composition of TAM® revealed in this study is good compared to the green and red seaweed extracts detected in previous studies, which reported levels of various minerals such as Mg, Na, K, Fe, Mn, Zn and Cu [70–72]. These results were in agreement with previous studies, which showed the beneficial effects of diluted seaweed extracts on plants, such as improved crop growth performance, nutrient contents, yield, and fruit quality [6,73].

GC-Mass analysis showed that TAM® extract contains different bioactive phytochemical compounds with many biological activities [45]. Methylated fatty acids and fatty acids represented about 83% of the total peak area of TAM®. Linoleic, γ-linolenic and oleic acid are the most abundant fatty acids found in seaweeds [23]. Biofertilizers in such fatty acids and their methyl ester have shown beneficial effects in antimicrobial and antioxidant activity [44]. In this context, seaweeds rich in fatty acids, minerals and polysaccharides stimulate the germination of many seeds, improve the quality of crop plants, enhance the resistance to climatic changes and insect pests, and prolong seeds and fruits preservation [74]. Phytol is one of the chlorophyll products that has been reported to have significant antioxidant effects [45,75]. Rhodopin, as a carotenoid compound in seaweeds, has antioxidant activity [49,51]. Nonadecane reported in many red, brown, and green seaweeds displayed antioxidant and antimicrobial activities [48–50]. This finding reflects the key role of these bioactive compounds as a protective effect against plant pathogens and invaders. Interestingly, TAM® showed new reported bioactive compounds, which for the first time were reported in seaweed extract [12] and utilized in the current study for the first time as plant foliar spray, such as milbemycin-oxime and 5-silaspiro[4.4]nona-1,3,6,8-tetraene,3,8-bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl. The milbemycin-oxime showed strong antiparasitic and insecticidal activities [52,53]. Moreover, 5-silaspiro[4.4]nona- is a phytobioactive compound and has two atoms of boron attached with one atom of silicon. Boron is an essential micronutrient required for the embryonic development and bone metabolism of plants [75,76]. However, many studies are still needed at this point.

Although the content of macro- and microelements in TAM® extract is lower than that used in the NPK control, its effect is almost better than the NPK control; this may be due to the fact that these elements are completely soluble in water and presented in the form of chelate that plants absorbed it efficiently over the soil fertilizer [77]. The enhancement of vegetative growth, yield, and bioactive ingredients of *E. vesicaria* was also reported earlier using different concentrations of commercial seaweed extract (Algamex) as a foliar spray on *E. vesicaria* [31]. Similar results were found using different foliar spray concentrations from the autoclaved cellular content of a blue-green alga, *Arthrospira platensis* (formerly *Spirulina platensis*), applied on *E. vesicaria* [60]. Yıldıztekın et al. [78] also recorded an enhancement in growth and plant yield when seaweed extracts from *Ascophyllum nodosum* were applied to *Capsicum annuum*. Applying red seaweed extracts of *Kappaphycus alvarezii* and *Gracilaria edulis* has been detected as plant growth and yield stimulants of wheat [79]. Applications of seaweed extracts have improved the total yield of *Phaseolus radiate L*. These results are overlapping those of Rathore et al. [80], in which applying seaweed extract (*K.*

alvarezii) induced growth, yield and nutrient utilization in soybean plants. Mola et al. [81] detected that biostimulant applications of seaweed extracts (*Ecklonia maxima*) increased the yield of baby leaf lettuce by 13.4% over non treated plants. Another study proved that the addition of acid green seaweed extracts of *Ulva lactuca* at low concentrations (0.2%) can significantly increase seed germination rates and fresh and dry weight production of mung bean compared to the control treatments [82]. The nonsignificant difference of most morpho-agronomic parameters and bioactive significant components in both cultivated years might be because cultivation time was the same in both years, as well as insignificant differences in the climatic conditions and soil parameters.

Interestingly, *E. vesicaria* grown under all three treatments of TAM®contained significantly higher amounts of total phenol and total flavonoid compounds as well as antioxidant activity compared to those obtained from the NPK control. Colla et al. [83] found that the application of brown seaweed (*Ecklonia maxima*) increased the yield and content of mineral, lycopene, total phenols and ascorbic acid as well as antioxidant activity of *Solanum lycopersicum*. Our results on TAM were consistent with the findings of Ali et al. [84], who observed that the foliar application of *Ascophyllum nodosum* (0.5%) enhanced the plant quality of tomato by 54% relative to the control. Earlier, Bell and Wagstaff [85] reported that flavonoids and phenolic compounds are the major phytochemicals found in different parts of *E. vesicaria*. Flavonoids in this plant are usually found with sugars as conjugates and typically occur in relatively large quantities [86]. Recently, El-Wakeel et al. [87] have studied the effects of natural flavonoid compounds from *E. vesicaria* to manage two annual weeds of *Pisum sativum* (Pea) plants. PCA is a useful analytical approach for illustrating the impact of environmental factors and genetic traits on the productivity and quality of plants [83,88,89]. In this study, the PCA score plot was divided into the TAM®-treated and NPK control plant (untreated). Most of the morphometric (total yield, number of leaves, and plant height) and phytochemical parameters (N, P, K, Chl. b, total phenols, and flavonoid content) of *E. vesicaria* under the treatments fell in PC1. Finally, NPK control plants had the lowest nutritional quality compared to that of the treatments.

Rocket salad, *E. vesicaria,* is a good source of polyphenols, which work as natural antioxidants [90]**.** In this study, it was found that *E. vesicaria,* treated with three TAM® concentrations or NPK chemical fertilizers, showed considerable antioxidant activity either by DPPH or phosphmolypedate methods (Figure 4). These findings were in agreement with Hassan et al. [60], who reported that foliar spray of *A. platensis* increased antioxidant activities of *E. vesicaria*. The Pearson correlation coefficient obtained for antioxidant activity with carotenoids and phenolics (Table 6) further confirmed the above findings. Earlier, Gutiérrez et al. [91] also reported that antioxidant activity and the phenolic contents in *E. vesicaria* correlated significantly. Interestingly, TAM® could also increase cucumber *Cucumis sativus* yield due to improving its chemical and physical traits related to immunity, productivity, and stress defense, under greenhouse conditions [47].

Many Arabian medicinal plants have been used as alternative cytotoxic natural sources throughout history [37]. Khoobchandani et al. [92] demonstrated that isothiocyanates found in *E. vesicaria* seed oil had anti-melanoma activity and played an essential role in inhibiting cancer cell proliferation. However, there is little information on the cytotoxic activity of *E. vesicaria* on lung carcinoma (A549) and hepatocellular carcinoma (HepG2). In the current study, among three different foliar spray concentrations of TAM®, 10% showed the highest cytotoxic effect (60.40%) with an IC_{50} value equal to 85.7 µg mL^{-1} against HepG2 only. These findings agreed with the results obtained by Hassan et al. [60]**,** who reported that foliar spray prepared from autoclaved cellular content of *S. platensis* could significantly increase cytotoxic activity by 61.3% against the hepatocellular carcinoma cell line (HepG2).

5. Conclusions

This study has shown that a new foliar spray formed of commercial seaweed extract (TrueAlgaeMax, TAM®) significantly improved the morpho-agronomic and bioactive properties of a Rocket salad, *E. vesicaria*, which were nicely comparable with those obtained

from the NPK control. Among the three TAM® treatments, 10% concentration resulted in the maximum influence on the most morpho-agronomic, mineral and bioactive compounds of *E. vesicaria*. Interestingly, TAM® showed new reported bioactive compounds, which for the first time were reported in these seaweed extracts and utilized in the current study as plant foliar spray, such as milbemycin-oxime and 5-silaspiro[4.4]nona-1,3,6,8-tetraene,3,8-bis(diethylboryl)-2,7-diethyl-1,4,6,9-tetraphenyl; however, many studies are still needed to determine the actual effects of these phyto-bioactive compounds on plants. Eventually, *E. vesicaria* grown with 10% TAM® showed the highest antioxidant activity and cytotoxic activity (60.40% with an IC_{50} value 85.7 μg mL^{-1}) against the hepatocellular carcinoma cell line (HepG2). This study shows that TAM® may be a feasible tool for improving the growth and yield of Rocket salad plants. Furthermore, the product's preparation is easy, has no side effects and has much potential to be an alternative to NPK fertilizers. However, more research is needed to enable the adoption of TAM®.

6. Patents

Seaweed extract (TrueAlgaeMax, TAM®) is a patent submitted at the Egyptian Patents Office, Academy of Scientific Research and Technology (submission No.: 2046/2019).

Author Contributions: Conceptualization, S.M.H., M.A., A.A.F.S., and M.E.E.; methodology M.A., S.M.H., and A.A.F.S.; software, M.E.E.; validation, S.M.H., M.A. and M.E.E.; formal analysis, S.M.H. and M.A.; investigation, M.E.E.; resources, M.A., H.A.H., W.F.A., and A.G.; data curation, H.A.H.; writing—original draft preparation, S.M.H., A.A.F.S., M.E.E. and M.A.; writing—review and editing, M.A. and M.E.E.; visualization, S.M.H., M.A., H.A.H., W.F.A., A.G., and M.E.E.; supervision, S.M.H., M.A., and M.E.E.; project administration, M.A.; funding acquisition, M.A., W.F.A., A.G., and H.A.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Two-Way Analysis of Variation (ANOVA) Performed for Different Parameters.

Parameters	Factors	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Plant height	Years	57.01	1	57.01	656.80	0.00
	Treatments	231.76	3	77.25	890.00	0.00
	Interaction	24.48	3	8.16	94.01	0.00
Number of leaves	Years	2.05	1	2.05	6.04	0.03
	Treatments	4.45	3	1.48	4.36	0.05
	Interaction	1.78	3	0.59	1.75	0.20

Table A1. *Cont.*

Parameters	Factors	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Dry matter	Years	0.06	1	0.06	0.19	0.67
	Treatments	14.17	3	4.72	13.89	0.00
	Interaction	0.15	3	0.05	0.15	0.93
Total yield	Years	0.00	1	0.00	0.01	0.92
	Treatments	0.90	3	0.30	3.46	0.04
	Interaction	0.22	3	0.07	0.85	0.49
Chl. a	Years	0.00	1	0.00	0.35	0.56
	Treatments	0.22	3	0.07	16.92	0.00
	Interaction	0.03	3	0.01	2.53	0.09
Chl. b	Years	0.09	1	0.09	0.18	0.68
	Treatments	54.99	3	18.33	39.03	0.00
	Interaction	0.95	3	0.32	0.67	0.58
Carotene	Years	0.00	1	0.00	0.00	0.98
	Treatments	4.57	3	1.52	42.81	0.00
	Interaction	0.10	3	0.03	0.89	0.47
Total phenols	Years	19.15	1	19.15	0.42	0.52
	Treatments	3485.78	3	1161.93	25.68	0.00
	Interaction	62.40	3	20.80	0.46	0.71
Nitrogen	Years	0.00	1	0.00	0.70	0.42
	Treatments	0.16	3	0.05	15.60	0.00
	Interaction	0.00	3	0.00	0.12	0.95
Phosphorus	Years	0.00	1	0.00	0.05	0.82
	Treatments	0.01	3	0.00	1.00	0.42
	Interaction	0.00	3	0.00	0.20	0.90
Potassium	Years	0.00	1	0.00	0.10	0.75
	Treatments	4.80	3	1.60	137.48	0.00
	Interaction	0.01	3	0.00	0.23	0.88
Total flavonoids	Years	693.38	1	693.38	0.86	0.37
	Treatments	9,586,325.46	3	3,195,441.82	3950.88	0.00
	Interaction	507.46	3	169.15	0.21	0.89
DPPH	Years	3.11	1	3.11	0.60	0.45
	Treatments	1078.40	3	359.47	69.72	0.00
	Interaction	10.15	3	3.38	0.66	0.59
Total antioxidants	Years	8.86	1	8.86	0.23	0.64
	Treatments	256.11	3	85.37	2.23	0.12
	Interaction	30.36	3	10.12	0.26	0.85

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