Evaluation of the effects of *Chlorella vulgaris*, *Nannochloropsis salina*, and *Enterobacter cloacae* on growth, yield and active compound compositions of *Moringa oleifera* under salinity stress

Munirah F Al Dayela, Fadia El Sherif

*Department of Biological Sciences, Faculty of Science, King Faisal University, Saudi Arabia*
*Department of Horticulture, Faculty of Agriculture, Suez Canal University, Egypt*

**Abstract**

Application of *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* has been reported to improve the growth of multiple plant species. *Moringa oleifera* is a medicinal plant found in Saudi Arabia. Its leaves, flowers and fruit have been used as food. *Moringa oleifera* is rich in rutin and gallic acid and many other bioactive compounds, which collectively contribute to its demonstrated range of pharmacological activities. In Saudi Arabia, the semi-arid and arid weather presents a significant challenge to agriculture. High salinity in cultivated land is a particular threat. We applied *Chlorella vulgaris*, *Nannochloropsis salina*, and *Enterobacter cloacae* at multiple salinites to *Moringa oleifera* to investigate their effects on the growth, yield, and photosynthetic pigment content. We also examined possible changes in the phytochemical composition. The application of *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* enhanced plant growth and yield, while inhibition was observed at high (6000 ppm) salinity. The presence of *Chlorella vulgaris* and *Nannochloropsis salina* altered plant growth and yield and rutin and gallic acid content of *Moringa oleifera* plants grown in saline conditions. Microalgae species were recommended for use as a bio-fertiliser alternative to mainstream synthetic fertilisers.

**1. Introduction**

Biotic and abiotic stresses continue to affect crop production and productivity adversely. Damage from these stresses is responsible for massive economic losses worldwide. Salinity is one of the main abiotic environmental stresses (Safarnejad, 2004; Schwabe et al., 2006). The rise in arable land salinisation is likely to have negative global consequences (Hasegawa et al., b, 2000a; Zhu, et al., 2006). The rise in arable land salinisation is likely to have negative global consequences (Hasegawa et al., b, 2000a; Zhu, et al., 2006)

In Saudi Arabia, the semi-arid and arid climate presents great challenges to agriculture. Increased salinity on cultivated land is becoming a major threat. Different approaches such as the adoption of salt-tolerant cultivars and different agricultural practices to mitigate the negative effects of salinity on plant growth and yield have been investigated.

*Moringa oleifera* Lam, a widely cultivated plant of the Moringaceae family, is commonly known as the drumstick or ben oil tree (Verdcourt 1985). It is a fast-growing soft-wooded tropical perennial tree with a long history of traditional medical and culinary uses. It is widely grown in India, the Philippines, Sudan, South Africa, tropical Asia, the Caribbean and the Pacific Islands. It is ideal for cultivation in Saudi Arabia, as it is extremely drought tolerant and is widely cultivated in arid and semi-arid regions (Stephenson and Fahey, 2004; Galvez Tan and Galvez Tan, 2008; Mridha, 2015).

The *Moringa* tree produces nutrients and antipyretic, anti-inflammatory, antispasmodic, diuretic, anti-hypertensive, anticholesterol, antioxidant and antidiabetic compounds (Guevara et al., 1999; Bennett et al., 2003; Khanuja et al., 2005; Anwar et al., 2007; Shanker et al., 2007; Kasolo, 2010; Mbitkay, 2012; Jung and Pandey, 2014; Anwar et al., 2007). Various phenolic acids...
and flavonoid compounds such as rutin and gallic acid have also been found in Moringa (Alam et al., 2020). Rutin has shown a range of pharmacological activities, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardio-protective (Ganeshpurkar and Saluja, 2017; Javed et al., 2012; Richetti et al., 2011). Gallic acid has potential preventive and therapeutic effects in many conditions in which oxidative stress has been implicated, including cardiovascular diseases, cancer, neurodegenerative disorders and in ageing (Kaur et al., 2005; Nikolic, 2006; Singh et al., 2018). The moringa tree is also salt tolerant, but the salinity level of this plant affects growth and yield up to 8.0 dS/m (Radovich, 2010; Nouman et al., 2012; Hussein and Abu-Baker, 2013; Fatima et al., 2018).

Excessive use of synthetic chemical fertilisers may have adverse environmental effects. Thus alternative sources, namely biofertilisers, have been proposed to replace chemical fertilisers partially or fully. These biofertilisers are cost-effective and environmentally friendly. Enterobacter spp. and microalgae such as Chlorella vulgaris and Nannochloropsis salina have been identified as plant growth enhancers, as they have many growth promoters and have played a pivotal role in building and sustaining soil fertility, thereby raising the growth and yield of several agricultural crops (Deepa et al., 2006; Singh et al., 2018). The moringa tree is also salt tolerant, and flavonoid compounds such as rutin and gallic acid of diluted seawater irrigated Moringa oleifera.

2. Material and methods

2.1. Sources of plant, algae and bacteria

Moringa oleifera Lam seeds were collected from the Department of Medicinal and Aromatic Plants, Institute for Horticultural Science, Agricultural Research Center (ARC), Giza, Egypt. Fresh C. vulgaris and N. salina alga were obtained from the Fisheries Research Centre, King Faisal University, Kingdom of Saudi Arabia. Enterobacter cloacae was obtained from the Microbiology Laboratory, Department of Biological Sciences, Faculty of Science, King Faisal University.

2.2. GC/MS analysis

The GC/MS analyses of C. vulgaris and N. salina were performed at the Department of Clinical Studies, College of Veterinary Medicine, King Faisal University. Both air-dried algae samples were extracted with methanol, according to McKenney et al. (2016). The methanol extracts were analysed by gas chromatography coupled with mass spectrometry (GC/MS-QP 2010 Plus), equipped with an auto-sampler AOC-20i (Shimadzu, Kyoto, Japan). Separation was performed with a 30 m × 0.25 mm × 0.1 μm RTX®-551MS capillary column (Restek, Bellefonte, PA, USA). The stationary phase was composed of 5% diphenyl and 95% dimethylpolysiloxane and high purity helium gas (99.9999%) used as a carrier gas. The helium gas flow rate, sample volume, and temperature program setting were as described by El Sherif et al. (2020). The computation of composition was according to Lee et al. (2018) with slight modification.

2.3. Moringa plant cultivation and algal/bacterial treatment

The experiment was conducted under greenhouse conditions at the King Faisal University Agriculture and Veterinary Research and Training Centre. The temperature was maintained between 32 and 36 °C, the relative humidity was 47–56%, and the average photoperiod was 14 h. Seeds were sown on 1 March 2019 in germination trays (depth of 1.0–2.0 cm.) filled with a moist mixture of (1:1 v/v) of sand and peat moss. After one month, the seedlings were transplanted into 20 cm diameter plastic pots with a depth of 15 cm, containing 4.5 kg of a moist mixture of (1:1 v/v) of sand and peat moss per pot. In a split-plot pattern, pots were arranged with two factors: C. vulgaris, N. salina and E. cloacae strain MSR1 OD (500) as a sub-factor and saline water as a major factor (0, 3000 and 6000 ppm) with 20 pots per treatment (one plant/pot). The cell suspensions of C. vulgaris and N. salina were adjusted to 1.5 × 107 cells/ml. Suspensions were mixed with either fresh or saline water at 3000 or 6000 ppm salinity to achieve a concentration of 0.4% (v/v) and applied to the pots at the beginning of the experiment. Fresh water (864 ppm) (Table 1) was used as a control. Seawater (45,000 ppm) from the Arabian Gulf, Damdam, Saudi Arabia, was diluted with fresh tap water (Table 1) to prepare saline solutions of 3000 and 6000 ppm salinity. An electrical conductivity meter (EcoSense A EC300) was used to measure the salinity of all solutions. Throughout the experimental period, plants were irrigated with corresponding salinity treatments to raise the soil water holding capacity. A pH meter (CIRISON Simple 20) was used to measure the pH. Various agricultural activities, such as weeding, were performed as recommended.

2.4. Vegetative growth

The plants were harvested after eight months from the date of their planting. Tree growth parameters such as plant height (cm), stem diameter (mm), number of leaves/plant (n), and leaves, roots and stem dry weight (g) of 10 random plants selected from each treatment group were recorded.

2.5. Chlorophyll pigments determinations

The plant pigments [chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoid] were extracted with 80% acetone from the 3rd bottom fresh leaf of 4 randomly selected 8-month-old moringa trees. Based on methods in A.O.A.C (1984), these pigments were measured spectrophotometrically and then estimated on a fresh weight basis as mg/100 g.

2.6. Determination of sodium and potassium concentrations in leaves

Plant leaves sampled 8 months after replanting were dried at room temperature and the air-dried matter was ground and digested, according to Piper (1947). Sodium and potassium were determined using atomic absorption flame photometry (3300), according to Wilde et al. (1985).

2.7. HPLC method for determination of rutin and chlorogenic acid

2.7.1. Instrumentation

The contents of rutin and gallic acid were determined from the air-dried samples of leaves and root per each treatment using the Waters 2690 Alliance HPLC system (USA) equipped with a Waters 996 photodiode array detector.

2.7.2. Materials and reagents

Authentic standards of rutin and gallic acid were obtained from Sigma-Aldrich. A rutin stock solution of 2 mg/ml in methanol was prepared and diluted to obtain standard solutions of 900 μg/ml, 750 μg/ml, 600 μg/ml, 450 μg/ml and 300 μg/ml. A gallic acid stock solution of 2 mg/ml in methanol was prepared, and 5 serial dilutions were prepared in concentrations of 1000 μg/ml, 800 μg/ml,
600 µg/ml, 400 µg/ml and 200 µg/ml. Each of the dilutions was filtered using a 0.22 µm syringe filter, and 10 µL were injected.

2.7.3. Sample preparation

The extracts were prepared from the dried samples by ultrasonic-aided extraction with methanol. Different weights of

---

**Table 1**

Chemical content of the irrigation water.

| Salinity Level (ppm) | Cations (meq L\(^{-1}\)) | Anions (meq L\(^{-1}\)) | SAR |
|----------------------|---------------------------|--------------------------|-----|
|                      | Ca\(^{2+}\) | Mg\(^{2+}\) | Na\(^{+}\) | K\(^{+}\) | CO\(_3\)\(^{2-}\) | HCO\(_3\)\(^{-}\) | SO\(_4\)\(^{2-}\) | Cl\(^{-}\) |
| 864                  | 5.72     | 2.02     | 7.27     | 0.38   | 0.28     | 2.68     | 4.03     | 8.4     | 3.43     |

**Table 2**

Phytochemical composition of methanol extracts from *Chlorella vulgaris* and *Nannochloropsis salina* by GC MS.

| Algae species          | RT    | Area   | Area% | MF    | MW     | Compound Name                                           |
|------------------------|-------|--------|-------|-------|--------|---------------------------------------------------------|
| *Chlorella vulgaris*    | 7.694 | 6426   | 3.75  | 57    | C2H3NO | Isocyanic acid, methyl ester                          |
| 17.391                 | 10062 | 5.87   | 102   | C3H10O2 | Capric acid methyl ester                                         |
| 18.764                 | 1751  | 1.02   | 194   | C3H6N2O4 | p-beta-Dinitrostyrene                                        |
| 19.402                 | 30187 | 17.61  | 268   | C7H32O2 | 9-Hexadecenoic acid, methyl ester, (Z)- $Methyl palmitoleate |
| 19.528                 | 110359| 64.36  | 270   | C17H34O2 | Palmitic acid, methyl ester                                  |
| 21.495                 | 2446  | 1.43   | 98    | C5H6O2  | Vinyl acrylate                                             |
| 21.825                 | 509   | 0.3    | 207   | C7H17N3O | p-Mentha-6,8-dien-2-one, semicarbazone                      |
| 22.008                 | 505   | 0.29   | 207   | C7H4F3O3 | p-Cresol, 2-nitro-alpha-alpha-alpha-trifluor                |
| 22.326                 | 9199  | 5.37   | 277   | C14H12ClNO | 2H-Indol-2-one, 1-(2,6-dichlorophenyl)-1,3-dihydro           |
| *Nannochloropsis salina* | 7.701 | 735    | 0.31  | 57    | C2H3NO | Isocyanic acid, methyl ester                          |
| 17.39                  | 14795 | 6.19   | 186   | C11H22O2 | Capric acid methyl ester                                           |
| 18.76                  | 1789  | 0.75   | 194   | C3H6N2O4 | p-beta-Dinitrostyrene                                        |
| 19.401                 | 50496 | 21.13  | 268   | C7H32O2 | 9-Hexadecenoic acid, methyl ester, (Z)- $Methyl palmitoleate |
| 19.628                 | 148332| 62.08  | 270   | C17H34O2 | Palmitic acid, methyl ester                                  |
| 21.496                 | 15416 | 6.45   | 111   | C7H13N  | 5-Methylhexanenitrile                                       |
| 22.324                 | 6881  | 2.88   | 242   | C14H7ClO2 | beta-Chloroanthraquinone                                    |
| 34.425                 | 504   | 0.21   | 207   | C11H17N3O | Imidazole, 2-bromo-4-methyl-5-nitro                      |

**Fig. 1.** Components identified in the methanol extracts from (a) *Chlorella vulgaris* and (b) *Nannochloropsis salina* by GC/MS analysis.
Each sample were combined with 50 ml methanol in conical flasks and sonicated for 30 min. The solvent was collected and replaced with 50 ml of fresh methanol every day for three consecutive days to ensure complete extraction before evaporating the methanol using a rotary evaporator at 40°C to obtain dry residue for each sample. Complete extraction was confirmed by thin-layer chromatography and high-performance liquid chromatography. For the HPLC analysis, a known weight of the residue was dissolved in 5 ml of the mobile phase in a volumetric flask. The contents of each flask were shaken vigorously for 10 min, then sonicated for 15 min before filtrated through a 0.45 μm disposable filters. Before injection, the sample was filtered with a 0.22 μm syringe filter. A sample of 10 μL was then injected, and the concentrations of rutin and gallic acid were calculated.

2.7.4. HPLC analysis conditions

The HPLC separation and quantitation were performed with a Column C18 Kromasil: 4.6 × 150 mm, 5 μm ODS column (Waters, USA). The mobile phase was prepared by mixing 0.1% phosphoric acid in water and acetonitrile in a ratio 5:95 v/v. The flow rate was 1 ml/min. All determinations were performed at ambient temperature (25 °C), Wavelength: 280 nm. The mobile phase was filtered using 0.45 μm membrane filter (Millipore, Milford, MA) and degassed by vacuum prior to use.

2.8. Statistical analysis

The data from all measurements were analysed using the Statistica 6 program ANOVA/MANOVA (StatSoft, 2001). The mean differ-

---

### Table 3

Effect of sea water concentrations, *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* on stem diameter, leaves number and plant height of *Moringa oleifera* plants.

| Algae and bacteria | Salinity (ppm) | Stem diameter (mm) | Leaves number (n) | Plant height (cm) |
|--------------------|----------------|--------------------|------------------|------------------|
| (a) Effect of alga and bacteria | | | | |
| Control | 11.09 b | 18.71 bc | 129.43 c | 129.43 c |
| *Chlorella vulgaris* | 17.04 a | 25.57 a | 191.64 a | 191.64 a |
| *Nannochloropsis salina* | 16.01 a | 26.46 a | 195.23 a | 195.23 a |
| *Enterobacter cloacae* | 12.13 b | 13.50 c | 149.43 b | 149.43 b |
| (b) Effect of sea water concentrations | | | | |
| Control | 18.99 a | 24.40 a | 231.15 a | 231.15 a |
| 3000 | 16.66 b | 26.18 a | 196.74 b | 196.74 b |
| 6000 | 6.27 c | 11.10 b | 68.00 c | 68.00 c |
| (c) The interaction between sea water concentration, alga and bacteria | | | | |
| Control | 19.26 ab | 21.00 b | 227.80 ab | 227.80 ab |
| *Chlorella vulgaris* | 19.45 ab | 22.75 b | 237.00 a | 237.00 a |
| *Nannochloropsis salina* | 17.94 ab | 46.20 a | 219.80 ab | 219.80 ab |
| *Enterobacter cloacae* | 19.48 a | 19.33 b | 237.20 a | 237.20 a |
| Control | 14.75 cde | 39.25 a | 168.25 c | 168.25 c |
| *Chlorella vulgaris* | 17.73 abc | 22.25 b | 211.75 ab | 211.75 ab |
| *Nannochloropsis salina* | 16.88 bcd | 25.40 b | 206.60 b | 206.60 b |
| *Enterobacter cloacae* | 16.92 bcd | 21.17 b | 211.00 b | 211.00 b |
| Control | 0.00 f | 0.00 c | 0.00 e | 0.00 e |
| *Chlorella vulgaris* | 14.44 de | 20.40 b | 132.6 d | 132.6 d |
| *Nannochloropsis salina* | 13.25 e | 15.50 b | 157.75 cd | 157.75 cd |
| *Enterobacter cloacae* | 12.13 e | 13.50 c | 149.43 b | 149.43 b |

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.

### Table 4

Effect of sea water concentrations, *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* on dry weight of leaves, stem and root of *Moringa oleifera* plants.

| Algae and bacteria | Salinity (ppm) | Dry weight of leaves (g) | Dry weight of stem(g) | Dry weight of root (g) |
|--------------------|----------------|--------------------------|----------------------|----------------------|
| (a) Effect of alga and bacteria | | | | |
| Control | 3.22 b | 36.09 c | 5.66 b | 5.66 b |
| *Chlorella vulgaris* | 5.51 a | 70.58 ab | 10.57 a | 10.57 a |
| *Nannochloropsis salina* | 5.26 a | 79.29 a | 10.28 a | 10.28 a |
| *Enterobacter cloacae* | 3.71 b | 59.17 b | 11.96 a | 11.96 a |
| (b) Effect of sea water concentrations | | | | |
| Control | 5.12 a | 94.75 a | 12.98 a | 12.98 a |
| 3000 | 5.92 a | 71.37 b | 12.00 ab | 12.00 ab |
| 6000 | 2.04 b | 16.91 c | 4.43 b | 4.43 b |
| (c) The interaction between sea water concentration, alga and bacteria | | | | |
| Control | 4.6 cd | 61.70 cd | 8.50 bcd | 8.50 bcd |
| *Chlorella vulgaris* | 6.18 abc | 104.70 ab | 12.53 abc | 12.53 abc |
| *Nannochloropsis salina* | 5.36 abcd | 107.00 a | 10.22 ab | 10.22 ab |
| *Enterobacter cloacae* | 4.84 bcd | 104.17 ab | 17.7 ab | 17.7 ab |
| Control | 5.07 bcd | 49.18 de | 7.05 cd | 7.05 cd |
| *Chlorella vulgaris* | 6.95 a | 83.45 abc | 12.58 abc | 12.58 abc |
| *Nannochloropsis salina* | 6.38 ab | 82.24 bc | 18.17 a | 18.17 a |
| *Enterobacter cloacae* | 6.13 abc | 73.35 c | 8.40 bcd | 8.40 bcd |
| Control | 0.00 e | 0.00 f | 0.00 d | 0.00 d |
| *Chlorella vulgaris* | 4.22 d | 32.04 e | 7.44 cd | 7.44 cd |
| *Nannochloropsis salina* | 6.38 ab | 39.98 de | 13.53 abc | 13.53 abc |
| *Enterobacter cloacae* | 6.13 d | 73.35 c | 8.40 bcd | 8.40 bcd |

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.
### Table 5
Effect of sea water concentrations, *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* on chlorophyll a/b and carotenoid of *Moringa oleifera* plants.

| Algae and bacteria | Salinity (ppm) | Chl b (mg/100 g F.W.) | Chl a (mg/100 g F.W.) | Carotenoids (mg/100 g F.W.) |
|--------------------|----------------|-----------------------|-----------------------|-----------------------------|
| (a) Effect of alga and bacteria | | | | |
| Control | 82.06 b | 25.76 a | 100.68 b |
| *Chlorella vulgaris* | 124.19 a | 25.36 a | 126.38 a |
| *Nannochloropsis salina* | 132.14 a | 27.98 a | 150.76 a |
| *Enterobacter cloacae* | 103.30 ab | 25.35 a | 121.45 ab |
| (b) Effect of sea water concentrations | | | | |
| Control | 138.71 a | 35.75 a | 156.16 a |
| 3000 | 120.17 ab | 29.56 ab | 139.29 ab |
| 6000 | 72.39 b | 13.02 b | 79.00 b |
| (c) The interaction between sea water concentration, alga and bacteria | | | | |
| Control | 111.98 bc | 14.58 cd | 117.40 bc |
| *Chlorella vulgaris* | 125.57 abc | 23.54 bc | 133.97 abc |
| *Nannochloropsis salina* | 178.29 a | 44.83 a | 202.00 a |
| *Enterobacter cloacae* | 138.99 abc | 35.29 ab | 171.27 ab |
| Control | 131.61 abc | 32.45 ab | 162.35 ab |
| *Chlorella vulgaris* | 86.99 c | 32.12 abc | 87.43 c |
| *Nannochloropsis salina* | 154.86 ab | 37.68 ab | 176.62 ab |
| *Enterobacter cloacae* | 107.19 bc | 40.75 ab | 130.77 bc |
| Control | 0.00 d | 0.00 d | 0.00 d |
| *Chlorella vulgaris* | 160.02 ab | 29.37 abc | 174.32 ab |
| *Nannochloropsis salina* | 129.57 abc | 22.73 bc | 141.68 abc |
| *Enterobacter cloacae* | 169.19 a | 0.00 d | 169.19 a |

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.

### Table 6
Effect of sea water concentrations, *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* on (K% and Na%) contents of *Moringa oleifera* plants.

| Algae and bacteria | Salinity (ppm) | Na% | K% |
|--------------------|----------------|-----|-----|
| (a) Effect of alga and bacteria | | | |
| Control | 1.2306 a | 0.0236 b |
| *Chlorella vulgaris* | 1.0197 b | 0.0453 a |
| *Nannochloropsis salina* | 0.6995 c | 0.0498 a |
| *Enterobacter cloacae* | 0.7110 c | 0.0249 b |
| (b) Effect of sea water concentrations | | | |
| Control | 0.5110 b | 0.0353 ab |
| 3000 | 1.0825 a | 0.0456 a |
| 6000 | 1.1071 a | 0.0242 b |
| (c) The interaction between sea water concentration, alga and bacteria | | | |
| Control | 1.2709 c | 0.0277 c |
| *Chlorella vulgaris* | 1.0394 bcd | 0.0380 abc |
| *Nannochloropsis salina* | 0.9655 d | 0.0429 abc |
| *Enterobacter cloacae* | 1.0542 bcd | 0.0317 bc |
| Control | 1.2019 ab | 0.0389 abc |
| *Chlorella vulgaris* | 1.0591 bcd | 0.0469 abc |
| *Nannochloropsis salina* | 1.0000 cd | 0.04874 ab |
| *Enterobacter cloacae* | 1.1675 abc | 0.0514 ab |
| Control | 0.0000 e | 0.0000 d |
| *Chlorella vulgaris* | 1.2151 ab | 0.0483 ab |
| *Nannochloropsis salina* | 1.0049 cd | 0.0566 a |
| *Enterobacter cloacae* | 0.0000 e | 0.0000 d |

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.*

ence between the treatment groups was evaluated at a probability level of $p = 0.05$.

### 3. Results

This study is the first to assess the effects of *Chlorella vulgaris*, *Nannochloropsis salina* and *Enterobacter cloacae* on the growth, yield and phytochemical composition of *Moringa oleifera*.

#### 3.1. GC/MS analysis of *C. vulgaris* and *N. salina*

The phytochemical composition of methanol extracts from *C. vulgaris* and *N. salina* are shown in Table 2 and Fig. 1. Fatty acid methyl esters (FAME) such as capric acid and palmitic acid were found in the methanol extract of both alga species. The results confirm the prerogative of polyunsaturated fatty acids in their cellular content is rich and diverse.

#### 3.2. Growth components

The survival rate of explants was 100% in the control group and 3000 ppm seawater treatments. However, at 6000 ppm, the survival rate was 0.0% (data not shown). These data suggest that the *M. oleifera* under study could tolerate salinity up to 3000 ppm.

The stem diameter, leaf number and plant height under salt stress and *C. vulgaris*, *N. salina* and *E. cloacae* exposure and both treatments together are presented in Table 3. The results indicate that increased seawater concentration significantly decreased stem diameter and plant height, except for plants treated with 3000 ppm in terms of leaf number (Tables 3b, c).

Stem diameter and plant height increased in the plants treated with *E. cloacae*, but this increase was not significant (Table 3c). The leaf number significantly increased in the plants treated with *N. salina* (Table 3c).

In the 3000-ppm salinity treatment group, the stem diameter and height of plants exposed to *C. vulgaris* treatments were greater than the control (3000 ppm) treatment (Table 3c). Treatment of *Moringa* seedlings with 6000 ppm salinity led to the death of plants in the control group and *E. cloacae* treatments. In contrast, 100% of the plants treated with *C. vulgaris* and *N. salina* survived at 6000 ppm salinity. The highest stem diameter and leaf number were observed in plants treated with *C. vulgaris* (Table 3c).

The presence of *N. salina* enhanced the plant height under 6000 ppm salinity (Table 3c).

#### 3.3. Yield of *M. oleifera*

The dry weight of leaves, stems and roots of *M. oleifera* under salt stress, with or without *C. vulgaris*, *N. salina* and *E. cloacae* is given in Table 4. The data showed that treatment supplemented with *C. vulgaris*, *N. salina* and *E. cloacae* resulted in the highest dry weight of leaves, stems and roots compared to the control.
treatment \( (\text{Tables 4a, c}) \). In contrast, the presence of salinity stress (except that of 3000 ppm) significantly decreased the dry weight of stems and roots and increased the dry weight of leaves \( (\text{Table 4b, c}) \). \textit{Chlorella vulgaris}, \textit{N. salina} and \textit{E. cloacae} had stimulation effects on the dry weight of leaves, stems and roots in plants grown under 3000 ppm salinity stress \( (\text{Table 4c}) \). Under high salinity stress (6000 ppm), the dry weight of leaves increased in plants treated with \textit{C. vulgaris}. A higher dry weight of stems and roots was observed in plants treated with \textit{N. salina} \( (\text{Table 4c}) \).

### 3.4. Chemical analyses

#### 3.4.1. Sodium and potassium contents

The presence of all three species enhanced the chlorophyll \( a, b \) and carotenoid contents relative to the control \( (\text{Table 5c}) \). \textit{N. salina} treatment conferred greater enhancement effects on these parameters than the control, \textit{C. vulgaris} and \textit{E. cloacae} treatments \( (\text{Table 5a,c}) \). The control treatment had inhibitory effects as demonstrated in the lower chlorophyll \( b \) and carotenoid contents \( (\text{Table 5a, c}) \). In contrast, these variables decreased in response to

![HPLC chromatogram of the methanolic extract of Moringa oleifera leaves (a) rutin authentic compounds, (b) the control plants and (c) plants exposed to Chlorella vulgaris and 6000 ppm salinity.](image)
salt stress (Tables 5b, c). \( N. \) \textit{salina} treatment under 3000 ppm salinity resulted in the highest chlorophyll \( a \), \( b \) and carotenoid contents relative to the control, \( C. \) \textit{vulgaris} and \( E. \) \textit{cloacae} treatments (Table 5c). However, \( C. \) \textit{vulgaris} treatment led to a smaller increase in the above parameters compared to \( N. \) \textit{salina} treatment under 6000 ppm salinity.

Fig. 3. HPLC chromatogram of methanolic extract of \textit{Moringa oleifera} (a) gallic acid authentic compounds, (b) root from plant exposed to \textit{Nannochloropsis salina} + 6000 ppm salinity levels and (c) root of plant exposed to \textit{Enterobacter cloacae} treatment.
The data in Tables 6a and 6c show that Na⁺ content increased in plants under the control and 3000 ppm salinity treatments relative to that of C. vulgaris, N. salina and E. cloacae treatments, smaller Na⁺ content was observed in plants exposed to N. salina treatment (Tables 6a, c). An increase of salinity levels also increased the Na⁺ content in leaves (Table 6b). Under 6000 ppm salinity, the C. vulgaris treatment contained higher Na⁺ percentage than the N. salina treatment (Table 6c).

The N. salina treatment produced the highest K⁺ percentage in leaves, and the other doses caused an increase in K⁺ compared to the control (Table 6a, c). Increased salinity treatments up to 3000 ppm resulted in a decrease in K⁺ in the plants in the control and C. vulgaris treatments compared to N. salina treatment (Table 6a, c).

### 3.4.2. Results for the HPLC method

A new single, isocratic, selective reverse phase-liquid chromatographic method has been developed for quantification of the rutin and gallic acid in the extracts of different treatments (Table 6 and Figs. 2 and 3). The method allowed good separation and quantification of the rutin and gallic acid within 18.808 and 7.667 min, respectively. The HPLC method was selective for rutin and gallic acid components. It was able to detect rutin and gallic acid contamination in the complex natural extract with minimal interference from other compounds in the extract.

The effect of salt stress, with or without C. vulgaris, N. salina and E. cloacae on rutin and gallic acid accumulation in M. oleifera have not been previously reported.

Rutin was found in the leaf extract of M. oleifera plant only, and the absence of this compound in the root extract is consistent with a previous study (Alam et al., 2020). Data (Table 7a and c) showed that the presence of C. vulgaris, N. salina and E. cloacae enhanced the rutin content compared to the control treatment. C. vulgaris treatment conferred a higher enhancement effect on rutin content than the control, N. salina and E. cloacae treatments (Table 7a and c). However, plants irrigated with 3000 and 6000 ppm saltwater showed a decrease in rutin compared to the control (Table 7b). The highest rutin production (1.10871 mg/g extract) was obtained in plants treated with C. vulgaris and irrigated with 6000 ppm salinity. The total amount of gallic acid (mg/g extract) in the leaves and roots of M. oleifera are presented in Table 7 and Fig. 3a and 3b. Results indicated that the leaf sample accumulated a higher amount of gallic acid compared to the root sample (Table 7). The amount of gallic acid increased in the leaf samples of plants exposed to C. vulgaris and N. salina treatment, respectively (Table 7c). Under 3000 and 6000 ppm salinity levels, the gallic acid accumulated at higher amounts in the leaf and root samples of M. oleifera treated with C. vulgaris (Table 7c). Increased salinity levels led to a decrease in gallic acid in both leaf and root samples of M. oleifera (Table 7a).

### 4. Discussion

Our study indicated that C. vulgaris, N. salina and E. cloacae as microorganisms were good elicitors in enhancing growth and phytochemicals accumulation in M. oleifera plant grown under different salinity levels. In terms of stem diameter and plant height, E. cloacae was a better elicitor than C. vulgaris or N. salina. However, N. salina resulted in higher leaf number and photosynthetic pigments. The presence of C. vulgaris, N. salina and E. cloacae enhanced the leaf, stem and root dry weight of the plants. The plant growth-stimulating effect of E. cloacae is due to its ability to make organic nitrogen sources available to plants (Santoyo et al., 2016; White et al., 2018; Macedo-Raygoza et al., 2019). Microalgae and E. cloacae as biofertilisers have been previously shown to increase plant growth and yield of some crops (Ozdemir et al., 2016; Borham et al., 2017; White et al., 2018; Satheeswaran and Jun, 2020). Salinity level of 3000 and 6000 ppm showed some toxic effects, as seen in the inhibition of plant growth, yield, and contents of chlorophyll b and carotenoids. A negative relationship was previously demonstrated between the degree of salt stress and M. oleifera plant growth characters, i.e. the dry weight of roots, stems and leaves, which decreased as the salt concentration increased in the diluted seawater (Hussein and Abou-Baker, 2013; Soliman et al., 2017). This reduction could be resulted by the blockage or reduced activity of the transporters caused by the high level of Na⁺. As the result, there

---

**Table 7**

Effect of sea water concentrations, Chlorelle vulgaris, Nanochloropsis salina and Enterobacter cloacae on rutin and gallic acid contents of Moringa oleifera plants.

| Algae and bacteria | Salinity (ppm) | Rutin mg/g extract in plant leaf | Gallic acid mg/g extract in plant leaf | Gallic acid mg/g extract in plant root |
|--------------------|---------------|----------------------------------|---------------------------------------|----------------------------------------|
| (a) Effect of alga and bacteria | Control | 0.27810c | 0.12786 b | 0.09594 a |
| Chlorelle vulgaris | 3000 | 0.66961 a | 0.33943 a | 0.21732 a |
| Nanochloropsis salina | 4.9467b | 0.17938 ab | 0.035162 a |
| Enterobacter cloacae | 0.30724c | 0.09207 b | 0.014837 a |
| (b) Effect of sea water concentrations | Control | 0.50708a | 0.240399 a | 0.08969 a |
| Chlorelle vulgaris | 3000 | 0.424073a | 0.190564 a | 0.016644 a |
| Nanochloropsis salina | 6000 | 0.381042a | 0.12309 a | 0.019419 a |
| Enterobacter cloacae | Control | 0.44858bc | 0.28021 ab | 0.274176 a |
| Chlorelle vulgaris | 3000 | 0.576077bc | 0.40585 a | 0.0104592 b |
| Nanochloropsis salina | 6000 | 0.52910bc | 0.10452 ab | 0.040883 b |
| Enterobacter cloacae | Control | 0.47458bc | 0.171016 ab | 0.031255 b |
| Control | 3000 | 0.258114 cd | 0.103363 ab | 0.013653 b |
| Chlorelle vulgaris | 3000 | 0.46102bc | 0.33540 ab | 0.022305 b |
| Nanochloropsis salina | 6000 | 0.52997bc | 0.21829 ab | 0.018623 b |
| Enterobacter cloacae | Control | 0.44717bc | 0.105194 ab | 0.011256 b |
| Control | 6000 | 0.000000 d | 0.000000 b | 0.000000 b |
| Chlorelle vulgaris | 6000 | 1.10871a | 0.27705 ab | 0.04598 b |
| Nanochloropsis salina | 6000 | 0.50545bc | 0.215334 ab | 0.03169 b |
| Enterobacter cloacae | 6000 | 0.000000 d | 0.000000 b | 0.000000 b |

*Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test.*
is a K+ and Na+ imbalance in the plant (Soliman et al., 2015; El-Garhy et al., 2016). A decreased K+ content is a response commonly observed in plants under salt stress because K+ directly competes with Na+ for binding sites that are charged dependent (Chen and Yu, 2007). The results herein showed that the application of microalga limited toxic ion accumulation, thus increased K+ contents (Kusuryan and Can, 2020). Increased salinity levels decreased rutin and gallic acid content. A decrease in the bioactive compounds content of M. oleifera resulting from increased salinity levels has been reported by Anwa et al. (2006). The presence of C. vulgaris and N. salina promoted plant growth and yield as well as rutin and gallic acid content in M. oleifera plants grown under different salinity levels. Nanochloropsis spp. and C. vulgaris are beneficial microbroscopic species that can increase nutrient uptake, growth and abiotic stress tolerance in plants (Agwa et al., 2017; Faheed, F.A., Fattah, Z.A., 2008. Effect of Chlorella vulgaris as bio-fertiliser on growth parameters and metabolic aspects of lettuce plant. J. Agric. Soc. Sci. 4, 165–169. FBO, 2005. Global Network on Integrated Soil Management for Sustainable Use of Salt-affected Soils. FAO Land and Plant Nutrition Management Service, Rome, Italy. http://www.fao.org/ag/agl/aglp/en/salt). El-Garhy, H., Khattab, S, MahmoudMoustafa, M.A., Rania Abou Ali, Azeiz, AZ, Eltabawy, A, El Sherif, F., 2016. Eluciating the expression of callose synthase genes (CHS) enhanced the silymarin yield and induced salinity tolerance of Silybum marianum L. Plant Physiology and Biochemistry 108, 191–202. Han, X., Zeng, H., Bartocci, P., Fantozzi, F., Yan, Y., 2018. Phytohormones and Effects on Growth and Metabolites of Microalgae: A Review. Fermentation 4, 4–25. Hasegawa, P.M., Bressan, R.A., Paro, J.M., 2000a. The dawn of plant salt tolerance genetics. Trends Plant Sci. 5, 317–319. Hasegawa, P.M., Bressan, R.A., Zhu, J.-K., Bahnert, H.J., 2000b. Plant cellular and molecular responses to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology 51, 463–499. Hussein, M.M., Abou-Baker, M., 2013. Growth and mineral status of Moringa plants as affected by silicate and salicylic acid under salt stress. J. Int. Plant Soil Sci. 3 (2), 163–177. https://doi.org/10.5734/jipss/2014/6105. Javed, H., Khan, M.M., Ahmad, A., Vaibhav, K., Ahmad, M.E., Khan, A., Ashafaq, M., Hussein, M.M., Abou-Baker, M., 2013. Growth and mineral status of Moringa plants as affected by silicate and salicylic acid under salt stress. J. Int. Plant Soil Sci. 3 (2), 163–177. https://doi.org/10.5734/jipss/2014/6105. Jeong, B.-R., Chang, Y.K., 2015. Effects of overexpression of a bHLH transcription factor on biomass and lipid production in Chlorella vulgaris. Biofuels 8, 200.https://doi.org/10.1186/s13068-015-0386-9. Kaur, Swayamjot, Michael, Husheem, Arora, Saroj, Härkönen, Pirkko L., Kumar, Subodh, 2005. The in vitro cytotoxic and apoptotic activity of Triphala—an Indian herbal drug. J. Ethnopharmacol. 97 (2), 163–177. https://doi.org/10.1016/j.jep.2004.09.003. Kalfa, U., Bernstein, N., 1996. Root growth under Salinity Stress. In: Plant Roots – the Hidden Half. Marcel Dekker, New York, pp. 463–499. Kang, N.K., Jeon, S., Kwon, S., Koh, H.G., Shin, S.-E., Lee, B., Choi, G.-G., Yang, J.-W., Jeong, K.-R., Chang, Y.M., 2015. Effects of overexpression of a BHLH transcription factor on biomass and lipid production in Nanochloropsis spp. Algal Research. Algal Res. 6, 858–588. Kholss, E., Markes, A.E., Milonkovic, J., Montoro, O., Debbouze, R., Rad, C., 2018. Bioethanol fermenting effect of the non-photosynthetic species on wheat plants. J. Plant Growth Regul. 1–6. https://doi.org/10.1007/s00344-018-9870-7. Kusuryan, A., El-Garhy, H.A., 2016. In vitro assessment of salt tolerance in male poplar trees and associated molecular changes. J. Hortic. Sci. Biotechnol. 91 (6), 2–11. Kusuryan, A., Can, A.G., 2020. Effects of Microalgae (Chlorella vulgaris Beijerinck) on Seconder Metabolites and Antioxidative Defense System Improve Plant Growth and Salt Tolerance in Guaym (Cyamus tetragonoloba (L.) Taub.). Legume Res. - An Int. J. 43 (4), 56–60. Lee, Yongbok, Choi, Jaehyuck, Ahn, Soon Kil, Na, Jong-kuk, Shrestha, Krishna Kumar, Nguyen, Sanamang, Park, Sang Un, Choi, Sangho, Kim, Jae Kwang, 2018.
Quantification of arbutin in plant extracts by stable isotope dilution gas chromatography–mass spectrometry. Chromatographia 81 (3), 533–538. https://doi.org/10.1007/s10337-017-4346-5.

Macedo-Raygoza, C.M., Valdez-Salas, B., Prado, F.M., Prieto, K.R., Yamaguchi, L.F., Kato, M.J., Canto-Canché, B.B., Carrillo-Beltrán, M., Di Mascio, P., White, J.F., Beltrán-García, M., 2019. Enterobacter cloacae, an endophyte that establishes a beneficial symbiosis with banana plants and protects against the black Sigatoka pathogen. Front. Microbiol. 10, 804.

Mbikay, M., 2012. Therapeutic potential of Moringa oleifera leaves in chronic hyperglycemia and dyslipidemia: a review. Front. Pharmacol. 3, 24.

McKenney, Janet, Onenç, Sermin, Pala, Mehmet, Magusie, Julie, 2016. Supercritical carbon dioxide treatment of the microalgae Nannochloropsis oculata for the production of fatty acid methyl esters. J. Supercrit. Fluids 116, 264–270. https://doi.org/10.1016/j.supflu.2016.06.003.

Mritha, M.A.J., 2015. Prospsects of Moringa Cultivation in Saudi Arabia Journal of Applied Environmental and Biological Sciences 5(3), 39–46.

Nikolic, Katarina M., 2006. Theoretical study of phenolic antioxidants properties in reaction with oxygen-centered radicals. J. Mol. Struct. (Theochemistry) 773 (1-3), 95–105. https://doi.org/10.1016/j.theochem.2006.07.017.

Nounan, W., Siddiqui, M.T., Basra, S.M.A., Khan, R.A., Gull, T., Olson, M.E., Munir, H., 2012. Response of Moringa oleifera to saline conditions. Int. J. Agric. Biol. 14 (5), 557–562.

Oancea, F., Veleva, S., Fătu, V., Mincea, C., Ilie, L., 2013. Micro-algae based plant biostimulator and its effect on water stressed tomato plants. Romanian J. Plant Prot. 6, 104–117.

Ordög, V., Stirk, W.A., Lenobel, R., Bancírová, M., Strnad, M., van Staden, J., Szigeti, J., 2012. Therapeutic potential of Moringa oleifera L. by reverse phase HPLC. Chromatographia 81 (3), 533–538. https://doi.org/10.1016/j.chroma.2012.05.025.

Özdemir, S., Sukatar, A., Oztekin, G.B., 2016. Production of fatty acid methyl esters. J. Supercrit. Fluids 116, 264–270. https://doi.org/10.1016/j.supflu.2016.06.003.

Pemmaraju, Deepak, Appidi, Tejaswini, Minhas, Gillipsie, Singh, Surya Prakash, Khan, Nooruddin, Pal, Mahadeb, Srivastava, Rohit, Rengan, Aravind Kumar, Shanker, K., Gupta, M.M., Santosh, K., Srivastava, S.K., Bawankule, D.U., Pal, A., Khana, S.P., 2007. Determination of bioactive nitrile glycoside(s) in drumstick (Moringa oleifera) by reverse phase HPLC. Food Chem. 105 (1), 376–382.

Singh, M.P., Avneet, G., Sisodia, S.S., 2018. Gallic acid: pharmacological promising lead molecule: a review. Int. J. Pharm. Phytochem. Res. 10 (4), 132–138.

StatSoft, 2001. STATISTICA Fur Windows (software-system fur Datenanalyse). StatSoft. 2001. STATISTICA Fur Windows (software-system fur Datenanalyse). StatSoft. 2001. STATISTICA Fur Windows (software-system fur Datenanalyse).