Chemical Methodologies

Journal homepage: http://chemmethod.com

Original Research Article

Determination of Dunaliella Salina Phenolic Compounds in *Laboratory Different Conditions*

Alireza Dolatyari, Orang Eyvazzadeh*, Leila Nateghi

Department of Food Science and Technology, Faculty of Agriculture, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran

ARTICLE INFO

Article history

Submitted: 2021-07-19
Revised: 2021-11-26
Accepted: 2021-12-03
Manuscript ID: CHEMM-2111-1394
Checked for Plagiarism: **Yes**
Language Editor:
Ermia Aghaie
Editor who approved publication:
Dr. Hamid Reza Arandiyan

DOI: 10.22034/chemm.2022.2.4

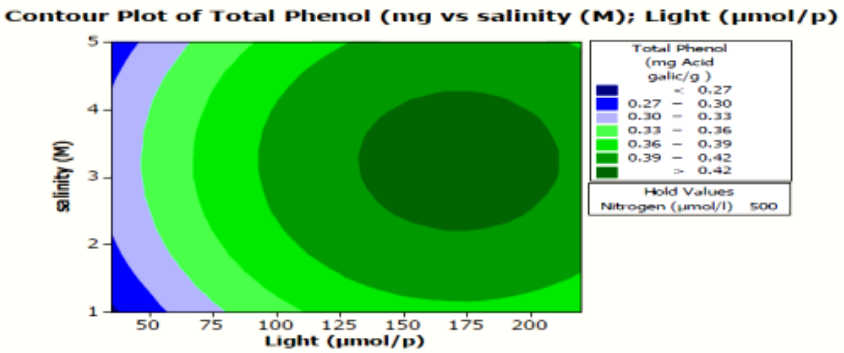
KEYWORDS

Algae
Dunaliella Salina
Phenolic compounds
Folin-Ciocalteo

ABSTRACT

Phenols are important natural compounds with antioxidant and other biological properties. Phenolic compounds have great advantages in food, chemistries, pharmaceutical and medical industries. Previous studies revealed that many phenolic compounds effectively scavenge hydroxyl and peroxyl radicals and prevent fats oxidation. The aim of this study was to optimize the laboratory different conditions including salinity (1, 3 and 5 M), light intensity (35, 127.5 and 220 µmol) and nitrogen source (200, 500 and 800 mmol/l) on the phenolic content of the algae. Therefore, 15 treatments were designed according to the Box-Behnken response surface methodology in 16 mini tabs. The results showed that laboratory different conditions (salinity, light intensity, and nitrogen content) had significant effect on the number of phenolic compounds in *Donaliella salina*. The highest amount of phenol, 0.43 gallic acid/g, was obtained at light intensity of 220 µmol photon, salinity of 3 M and nitrate concentration of 200 µmol/L. By optimizing the culture medium of *Dunaliella salina*, optimal light radiation conditions of 178 µmol photon, salinity of 3.26 M and nitrogen concentration of 327 µmol/L the number of phenolic compounds could be increased (0.46 mg gallic acid/g).

GRAPHICAL ABSTRACT



* Corresponding author: Orang Eyvazzadeh
✉ E-mail: orang_eyvazzadeh@yahoo.com

Introduction

Algae are organisms that contain chlorophyll and conduct photosythesis [1]. Since ancient times the importance of algae has been known for humans. In past, algae were used primarily as food and medicine. The Chinese and Romans were the first people who found the algae valuable and used them in different areas. The greatest role played by algae can be found in marine ecosystems. They are considered primary producers in such environments. For example, Iran, has special climatic conditions allowing algae to be widely distributed so that they can be exploited for manufacturing pharmaceuticals and food supplements and therefore there is no need to import such materials from other countries [2]. Algae are inexpensive products for human consumption. They are rich in protein and omega3 fatty acid that make them very important sources for their nutritional value. Today algae are extensively used in food and pharmaceutical industries. The main advantages of algae are easy storage and their use in foods processing. *Dunaliella salina* algae is rich in beta-catotene which developes natural color in the product. Various studies have shown that algae are rich in protein, amino acids, vitamins C and B_{12}, fructose, carotenoids such as beta-carotene, alpha-carotene, lutein, xanthine and cryptoxanthin. Given the recent drought in our country, cultivation of such algae, which does not require much water, is easily feasible [3]. The main components of this algae are carotenoids, glycerol, proteins, and vitamins which have anticancer and antioxidant properties [4]. It has also different bioactivities including anticancer, antiatherosclerosis, and antidiabetic activities, vision improvement, allergy prevention, and detoxification [5].

Dunaliella salina algae is a unicellular flagellate green alga which is rich in carotenoids and beta-caroten, has no cell wall and lives at 0-38˚C with high salt tolerance [6].

One of the advantages of using algae like *D. salina* is their rapid growth rate. Growth of algae occurs in lag, logarithmic, stationery and death phases. Duriug the lag phase, the cells do not increase in number. The logarithmic phase is the most important phase of algae growth when the number of cells increases rapidly. The logarithmic phase is followed by the stationary one in which the number of cells remains constant, and the cells store energy for survival. Death phase is the last phase of growth cycle where cells gradually decrease in number due to lack of energy.

Salinity tension caused by soil salinity, irrigation without adequate drainage, and water salinity may limit the growth of plants. Salinity tension is among the critical factors affecting the agricultural crops resulting in decreased crop yield. It is a serious threat to agricultural society which pose physiological and metabolic problems and decrease the growth, germination, strength, the quantity, and quality of the plant. The toxic effect of salt on the plants is greater than that of all other elements. Identification of salinity-tolerant cultivars is a proper strategy to prevent the crop losses. Salt tolerance comprises several complex processes. Soil salinity affects water content, pigmants, lipids, nutrients absorption as well as metabolism which are discussed in the present study. Algae are mainly found in supersaturated saline lakes therefore saline lakes such as Urmia, Qom and Maharloo provide the favorable conditions for algae cultivation [7].

The best source of nitrogen for *D. salina* is nitrate which its absorption depends on light, for example ammonium nitrate was reported to prevent the formation of beta-carotene [8].

Free radicals are either generated in the body or come from the environment causing damages to cellular structures such as DNA, proteins, lipids as well as cellular membranes and develope cancer and atherosclerosis. They are also the main responsible for food spoilage and toxic compounds production [9].

In recent years, the phenolic content of algae in different media has been investigated. The effect of colchicine on growth and some metabolites of *Dunaliella salina* microalgae [10]. Use of whey as a medium for *D. salina* culture antioxidant properties and total phenolic content of a marine diatom, Navicula clavata and green microalgae Chlorella marina and *Dunaiella salina* [11] and the effect of cadmium (Cd) on the growth and the phenolic content were studied.

Response surface methodology (RSM) comprises some mathematical and statistical techniques which provide optimum conditions in complex systems. The theoretical basis of RSM is a powerful tool for empirical optimization searching for a relationship between independent and dependent variables. BOX-Behnkem is a second-order independent design which does not contain complete or incomplete factorial designs. In such design the treatments conbinations are at the midpoints and center points not at the edges of the process space. The design are rotatable and each factor needs to have three surfaces [12].

The culture meduim conditions may greatly affect the nutrients of this alga and the optimization of the medium conditions aiming at increasing its nutritional value has not been studied yet. The objective of this study was to optimize the culture medium including salinity (1, 3, and 5 mol/L), light intensity (35, 127.5, and 220 µmol) and nitrogen content (200, 500, 800 mmol/L) and examine their effect on phenolic content of *Dunaliella salina* alga.

Methods and Materials

Water samples containing algae were collected in 1 L glass containers from different parts of Urmia Lake. The chemicals used in this study were sodium carbonate, copper sulfate, sodium tartrate, potassium tartrate and sodium hydroxide (Merck, Germany).

D. salina alga culture at different concentrations of salinity, nitrogen and light intensities

The water samples containing algae collected from Urmia Lake were transferred to foods laboratory of Department of health, rescue and treatment of I.R. Iran army ground forces. The samples were purified by agar plate and serial culture. The culture medium conditions included salinity (1, 3 and 5 M) and nitrogen (200, 500 and 800 µmol/L). The prepared samples were irradiated at 35, 127.5 and 220 µmol photon in germinator. Each sample was tested in tripilicate. To measure the growth rate of *D. salina* alga, cell counting was performed daily via sampling from three replications of each treatment. The antioxidant activity of *D. salina* was evaluated at different conditions [13].

Total phenolic content

Total phenolic content was meansured by Folin-Ciocalteu method. This method is based on electron transport and phenolic reduction capacity. External calibration was performed with different concentrations of gallic acid. Then 9 ml of A (1.0 ml sodium carbonate + 1.0 mL copper sulfatet + 1.0 ml sodium- and pottasium tartrate) were added, mixed and after 4 minutes 4.0 ml 0.5 M sodium hydroxide were added. The solution was kept at ambient temprature and then the absorbance was measured at 750 nm by spectrophotometer (UV Visible, USA). Total phenolic content was measured based on gallic acid and the drawn diagram [14].

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Data analysis

The effect of optimum culture medium (salinity, light intensity, nitrogen) on the phenolic content of *D. salina* alga were analysed by response surface (RS) software using Box-Behnken method at 5% probability level.

Findings

Variance analysis of RS model for phenolic content of D. salina alga under different culture conditions

The result of variance analysis of phenolic content of D.salina alga and Equation 1 in regrescion model for phenolic content of *D. salina* under different culture conditions (nitrogen, light and salinity) are presented in Table 1. As shown in Table 1, the predicted regression model was significant ($p \leq 0.05$). The explanation coefficient and modified explanation coefficient were R-Sq

=99.50 and R-Sq (adj) = 98.59, respectively suggesting the good fit of the model. The linear and quadratic effect of all three variables, salinity, light intensity and nitrogen content, on the phenolic content of *D. salina* alga were significant ($p<0.05$). The interactive effect of «nitrogen content and light intensity» on the phenolic content was significant ($p>0.05$). Given the value of F factor, the linear effect of light intensity followed by the quadric effect of light intensity were the greatest effect on the antioxidant content of *D. salina* (Equation 1).

Y= 0.416667-0.028750A+0.052500B+ 0.008750C-0.032083A2 0.054583B2-0.037083C2-0.015000AB-0.002500AC.

Table 1: Results of variance analysis of phenolic content of *D. salina* alga under different culture conditions (nitrogen content, light and salinity)

Source	Degrees of freedom	Regression coefficient	Sum of squares	Mean square	F-value	P-value
Regression constant	9	0.416667	0.47652	0.005295	109.54	0.000*
Linear effects	3	-	0.029275	0.009758	201.90	0.000*
Nitrogen	1	0.028750	0.006612	0.006612	136.81	0.000*
Light	1	0.052500	0.022050	0.022050	456.21	0.000*
Salinity	1	0.008750	0.000613	0.000613	12.67	0.016*
Qadratic effects	3	-	0.017452	0.005817	120.36	0.000*
Nitrogen×Nitrogen(A^2)	1	0.032083	0.002434	0.003801	78.63	0.000*
Light×Light(B^2)	1	0.054583	0.009940	0.011001	227.60	0.000*
Salinity×Salinity(C^2)	1	0.037083	0.005078	0.005078	105.05	0.000*
Interactive effect	3	-	0.000925	0.000308	6.38	0.037*
Nitrogen×light(A×B)	1	0.015000	0.000900	0.000900	18.62	0.008*
Nitrogen×salinity(A×C)	1	0.002500	0.000025	0.000025	0.52	0.504
Light×salinity(B×C)	1	0.000000	0.000000	0.000000	0.00	1.000
Residual error	5	-	0.000242	0.000048	-	-
Lack-of-fit	3	-	0.000175	0.000058	1.75	0.384
Pure error	2	-	0.000067	0.000033	-	-
Total	14	-	0.047893	-	-	-
R^2				99.50%		
R-sq(adj)				98.59%		

*Significant difference (P≤0.05)

Interactive effects (salinity×light), (salinity×nitrogen) and (nitrogen×light) on phenolic content of D. salina alga

The results of interactive and surface effects of salinity and light on the phenolic content of *D. salina* alga at constant nitrogen content of 500 µmol/L are given in Table 2. As shown in the Table, as the light intensity increased higher phenolic content was found as ≥ 0.42 mg gallic/g was observed at 140-210 µmol photon, salinity of 2.2-4.2 M and nitrogen constant concentration of 500µmol/L.

Figure 1 (b) shows the interactive effects of (salinity × nitrogen) on the phenolic content of *D.salina* algae at constant light intensity of 127.0 µmol photon. Phenolic content of ≥0.42 mg gallic acid/g was found at constant salinity of 2.6-3-9 M salinity, nitrogen content of 280-480µmol/L, and constant light intensity of 127.5 µmol photon.

Figure 1(c) shows the interactive effects of (nitrogen × light) on the phenolic content of *D. salina* algae at constant salinity of 3 M. Phenolic content of ≥0.42 mg gallic acid/g was observed at constant nitrogen content of 200-570 µmol/L, light intensity of 125-220 µmol photon and salinity of 3 M.

Measurement of phenolic cotent of D. salina under different conditions by tested and predicted method

Phenolic content of *D. salina* under different conditions by tested and predicted method is presented in Table 2. The results revealed that there was no significant difference between phenolic content of *D. salina* alga obtained under different conditions by tested and predicted methods.

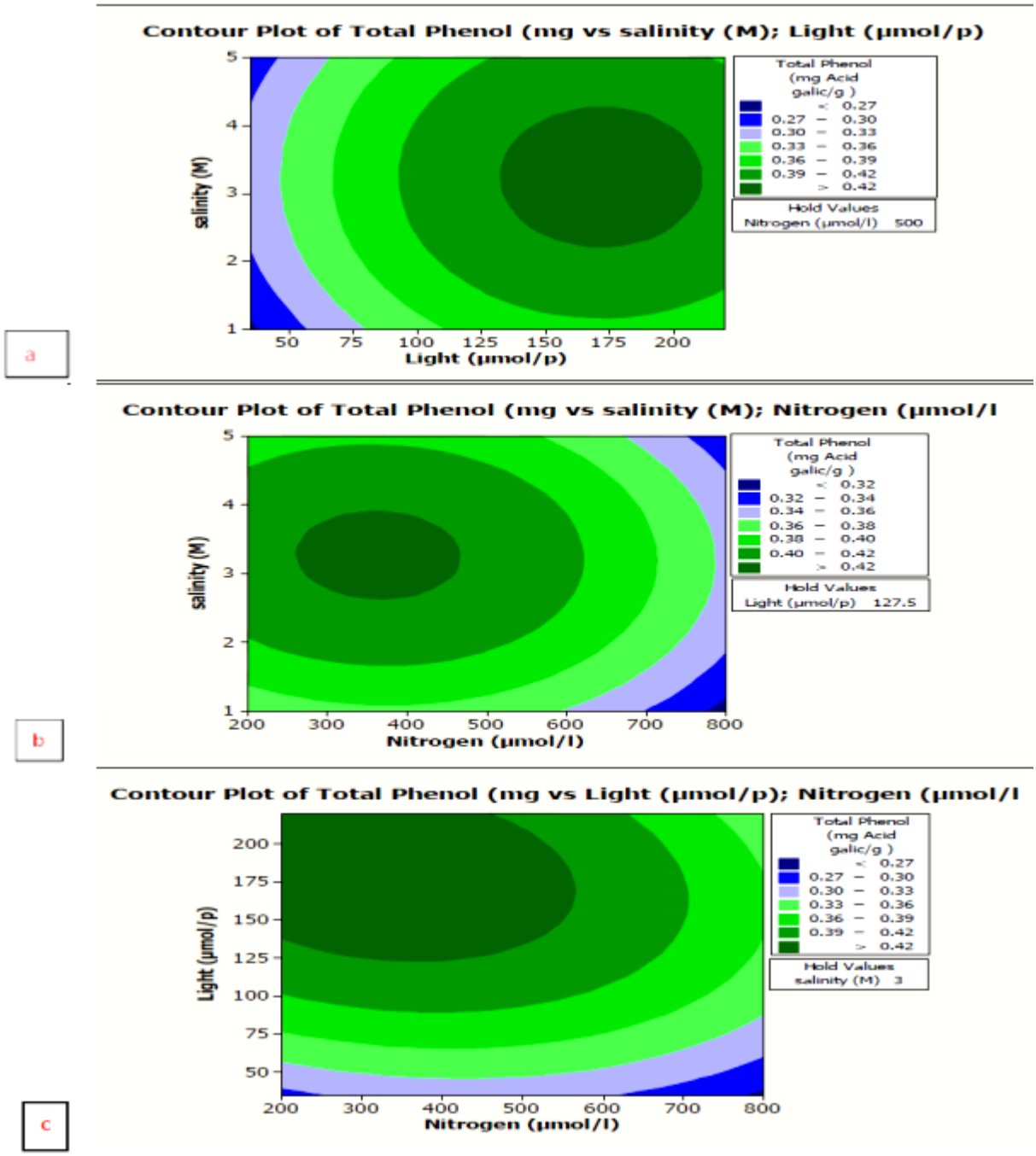

Figure 1: Interactive effects: (a) salinity × light on phenolic content of *D. salina* alga at nitrogen constant concentration of 500µmol/L, (b) salinity × nitrogen on phenolic content of *D. salina* alga at light constant intensity of 127.5µmol photon and (c) light × nitrogen phenolic content of *D. salina* at constant salinity of 3 M

As shown in Table 2, different culture conditions (salinity, light and nitrogen) had significant effect on the phenolic content of *D. salina* algae as the phenolic content ranged from 0.26 to 0.43 mg gallic acid/g. The highest phenolic content (0.42 mg gallic acid/g) was found at light intensity of 220 µmol photon, salinity of 3 M and nitrogen content of 200 µmol/L and the lowest phenolic content (0.26 mg gallic acid/g) was observed at light intensity of 35 µmol photon, salinity of 3 M and nitrogen content of 800 µmol/L.

Table2: Comparison of tested and predicted phenolic content of *D. salina* alga under different conditions

Treatment	Nitrogen (µmol/L)	Light (µmol/p)	Salinity (M)	Phenolic content (mg gallic acid/g)	Predicted phenolic content (mg gallic acid)	Residual
1	200	127.5	1	0.360	0.365	-0.005
2	500	220.0	1	0.370	0.369	0.001
3	200	220.0	3	0.430	0.426	0.004
4	500	127.5	3	0.420	0.417	0.003
5	500	35.0	1	0.270	0.264	0.006
6	200	127.5	5	0.390	0.388	0.003
7	500	127.5	3	0.420	0.417	0.003
8	800	127.5	1	0.310	0.312	-0.002
9	800	127.5	5	0.330	0.325	0.005
10	500	35.0	5	0.280	0.281	-0.001
11	500	127.5	3	0.410	0.417	-0.007
12	800	35.0	3	0.260	0.264	-0.004
13	500	220.0	5	0.380	0.386	-0.006
14	800	220.0	3	0.340	0.339	0.001
15	200	35.0	3	0.290	0.291	-0.001

Optimum conditions for phenolic content of D. salina alga under different conditions

Figure 2 demonstrates the optimum conditions for obtaining the maximum phenolic content of *D. salina* under different culture conditions. As illustrated in the Figure, the predicted maximum phenolic content of *D. salina* alga, 0.44 mg gallic acid/g with 100% utility was observed at light intensity of 178 µmol photon, salinity of 3.26 M and nitrogen content of 327 µmol/L. The in vitro optimal conditions for obtaining maximum predicted phenolic content were applied and the phenolic content of 0.46 mg gallic acid/g was obtained showing a significant ($p>0.05$) difference from the phenolic content predicted by response surface method.

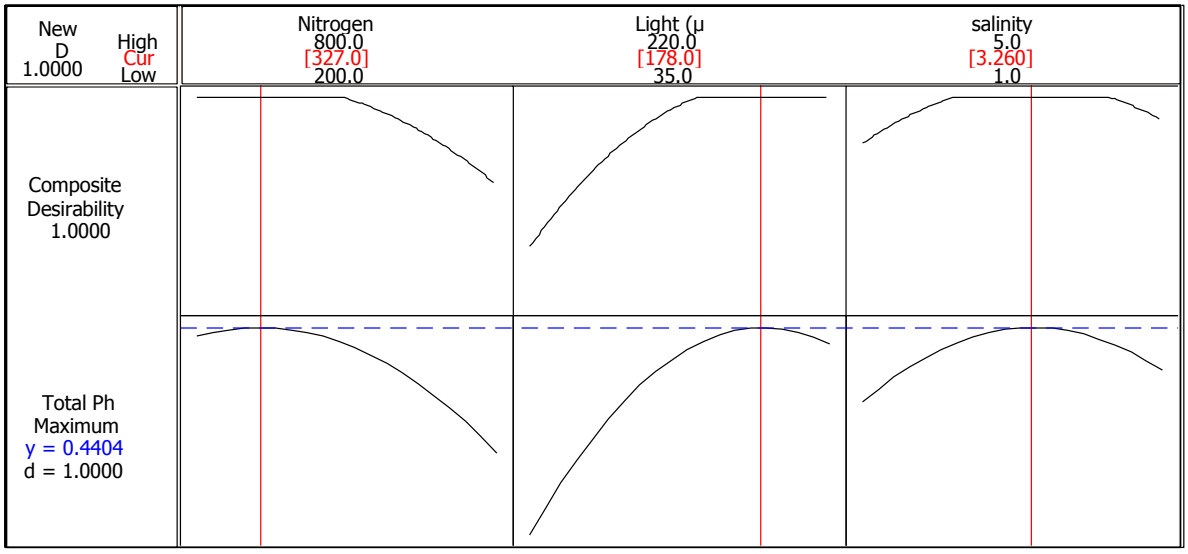

Figure 2: Optimum conditions for phenolic content of *D. salina* alga under different culture condition

Results and Discussion

Phenols are important natural compounds with antioxidant and other biological properties [16]. Phenolic compounds have great advantages in food, chemistries, pharmaceutical and medical industries.

Our results (Tables1 and 2) demonstrated that different culture conditions (salinity, light and nitrogen) had significant effect on the phenolic

content of *D. salina* alga as it ranged from 0.26 to 0.43 mg gallic acid/g. The highest phenolic content (0.43 mg gallic acid/g) was observed at light intensity of 220 µmol photon, salinity of 3 M and nitrogen content of 800 µmol/L.

The results (Table 1) showed that the phenolic content increased significantly as the light intensity increased. As the salinity increased from 1 to 3 M and nitrogen content increased from 200 to 500 µmol/L, the phenolic content increased significantly whereas it decreased significantly when the salinity increased from 3 to 5 M and the nitrogen content increased from 500 to 800 µmol/L. *D. salina* alga is the most resistant eukaryotic organism to salinity found in many saline euvironments such as saline lakes and swamps. The desirable salinity for *D. salina* growth is 3 M, however the critical points of salinity have been reported to be 0.5 and 5 M [17].

Previous studies revealed that many phenolic compounds effectively scavenge hydroxyl and peroxyl redicals and prevent fats oxidation. Phenolic content is increased at low temperatures. Low temperature along with light increase the oxidative stress by limiting photosynthetic enzymes thereby increasing the phenolics as photoprotective compounds [18]. Most algae increase their phenolic compounds when exposed to these environmental stress factors [19].

Because in this study the environmental tensions in the culture medium were avoided as much as possible, increased salinity caused a decrease in phenolic compounds production. The results of other studies agree with our results as Alirahimi *et al*., (2015) investigated the effect of colchicine on the growth and some metabolites of *D. salina* microalga and stated that different concentrations of colchicine caused significant ($P \leq 0.01$) difference in extracellular phenolic compounds [20].

In another study, Nabizadeh *et al*., (2020) examined the use of whey as culture medium for *D. salina* microalga. The ratios of Walne culture medium (0, 25, 50 µL), whey (0, 2.5, 5%) and time of incubation (0, 7, 14 d) to produce *D. salina* biomass as well as biochemical properties including phenolic compounds were evaluated. Their results showed that when the whey percentage increased, total phenolic content did not significantly ($P<0.05$) decreased. The use of whey as the culture medium for *D. salina* microalga was successful. Their result is consistent with our finding suggesting the favorability of the applied conditions, i.e. light (270 µmol photon), salinity (3 M) and nitrogen concentration (200 µmol/L) for the growth of *D.salina* alga and maximum production of phenolic compounds.

Hemalatha *et al*., (2013) measured total phenolic content of green algae Chlorella marina and *Dunaliella salina*. In *D. salina* the highest phenolic content was found for methanol followed by aceton and ethanol extracts.

Belghith *et al.,* (2016) studied the growth of *D. salina* affected by cadmium (cd) contamination. It was shown that Cd could stimulated the syntnesis of some secondary metabolites such as polyphenols and flavonoids. They play a crucial role in protecting *D. salina* against reactive onygen species (ROS) generated in the presence of Cd indicated by increased antioxidant capacity of *D. salina* exposed to Cd.

Conclusion

The present study aimed at investigating the optimum conditions for growth and phenolics compounds production in the culture medium of *D. salina*. The culture medium conditions were optimized with salinity (1, 3, and 5 M), light (35, 127.5, 220 µmol) and nitrogen content (200, 500, 800 µmol/L) parameters. The results showed that increase in nitrogen and salinity levels above a certain point reduced the phenolic content while increase in light intensity increased the phenolic content significantly. In the culture medium of *D.salina*, as the salinity increased from 1 to 3 M and nitrogen concentration increased from 300 to 500 µmol/L, the phenolic content increased significantly however it decreased as the salinity increased from 3 to 5 M and nitrogen level increased from 500 to 800 µmol/L. By optimizing the culture medium parameters including light (178 µmol photon), salinity (3.26 M) and nitrogen (327 µmol/L), the phenolic

content of *D. salina* alga increased from 0.26 to 0.46 mg gallic acid/g.

Acknowledgments
All thanks and appreciation to the pharmacist Tuqa S. Al-Amin for completing the biological applications.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions
All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest
We have no conflicts of interest to disclose.

ORCID
Leila Nateghi
https://www.orcid.org/0000-0001-8937-8728

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HOW TO CITE THIS ARTICLE
Alireza Dolatyari, Orang Eyvazzadeh, Leila Nateghi. Determination of Dunaliella Salina Phenolic Compounds in *Laboratory Different Conditions*, *Chem. Methodol.*, 2022, 6(2) 114-121
DOI: 10.22034/chemm.2022.2.4
URL: http://www.chemmethod.com/article_141170.html