Iranian Journal of Fisheries Sciences 21(2) 590-604 2022
DOI: 10.22092/ijfs.2022.126672

Research Article

Extraction of abscisic acid and gibberellin from *Sargassum muticum* (Phaeophyceae) and *Gracilaria corticata (*Rhodophyta*)* harvested from Persian Gulf

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Received: February 2021 Accepted: December 2021

Abstract

Phytohormones are present in seaweeds but little is known about occurrence and content of them in seaweeds of Persian Gulf. The aim of this study was extraction of abscisic acid and gibberellin in *Sargassum muticum* and *Gracilaria corticata*. The seaweeds were collected bimonthly over one year at Bushehr coasts, Persian Gulf, during a range of environmental conditions. We explored new HPLC method for extracting abscisic acid and gibberellin from the seaweeds. It was found that the lowest amount of abscisic acid in *Sargassum* and *Gracilaria* were 0% in several months and the highest were 20.667 and 66.20% in November, respectively. Maximum yield of gibberellin in *Sargassum* and *Gracilaria* occurred in July (58.561%) and May (84.467%), respectively. The highest *Sargassum* biomass obtained in January (679 g/m^2) and maximum biomass of *Gracilaria* was in March (423.33 g/m^2). The results showed that biomass of two algae had negative correlation with abscisic acid and positive with salinity. This is due to inhibitory effect of abscisic acid on growth. There was no significant correlation between gibberellin and biomass of the two algae. In this article we showed that phytohormones existing in seaweeds of Persian Gulf could be used in algae liquid fertilizer.

Keywords: Abscisic acid, Gibberellic acid, Algae, Persian Gulf

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Introduction

Extracts from algae have a variety of biotechnology activities such as anti-toxic, anti-bacterial, anti-viral, and anti-tumor. In addition to food and medicine (Hong *et al.*, 2007), marine algae are a source of future industrial materials, including algae liquid fertilizers (Thambiraj *et al.*, 2012) that include phytohormones or plant growth regulators. Plant growth regulators, including abscisic acid, gibberellins, auxins, and cytokines, are widely used in agriculture and pharmaceuticals (Sarkar *et al.*, 2002).

Plant hormones are vital for the normal functioning of plants. Their little quantities trigger basic developmental processes such as cell division, enlargement and differentiation, organ formation, seed dormancy and germination, leaf and organ senescence, and abscission (Dobrev *et al.*, 2005). Some phytohormones are known to be present in seaweeds as well as in terrestrial plants. However, a few studies reported on marine alga (Yalçın *et al.*, 2020). Cytokinins were reported from *Sargassum muticum*, *Porphyra perforata* and *Saccharina japonica*, Indole-3-acetic acid (IAA) in *Caulerpa paspaloides* and abscisic acid (ABA) in *Ulva lactuca*, *Ascophyllum nodosum*, three *Laminaria* species (Stirk *et al.*, 2009), gibberrellin in *Pyropia yezoensis* (Mikami *et al.*, 2016).

The first macroalgal extract introduced in late 1940s as Maxicrop for agricultural purposes (Dawes, 1982). Many macroalgae extracts are now available in liquid and powder forms, mainly brown algae as herbal stimulants, and are used as a mixture with soil or spray. These growth regulators can produce physiological responses in small amounts (Strike *et al.*, 2003). Under the brand name of Simplex and Acadian, *Ascophyllum* brown algae extract is now being imported to Iran as a growth stimulant, and algae fertilizer at exorbitant prices containing abscisic acid and gibberellic acid hormones (Verkleij, 1992; Sunarpi *et al.*, 2010).

Recently in Mexico, seaweed extracts were used as biostimulant, biofertilizer, metabolic enhancer, and root promoter in order to reduce ecosystem degradation and contamination of agricultural land. Some seaweed extracts like *Ascophyllum nodosum*, *Sargassum* spp., and *Macrocystis pyrifera* extracts are marketed as liquid biofertilizers or biostimulants (Hernández-Herrera *et al.*, 2018).

In Latin America *M. pyrifera*, *Gelidium robustum*, *Chondracanthus canaliculatus*, *Sargassum* spp., *Ulva lactuca*, and *Padina gymnospora*, are used as biostimulants, root promoters, biofertilizers, and growth stimulators (Hernández-Herrera *et al.*, 2014).

Featonby-Smith (1984) reported cytokinins seasonally in *Ecklonia* brown algae with the highest levels of zeatin in summer. Strike *et al.* (2009) extracted cytokinin, auxin, and abscisic acid from *Ulva* and *Dictyota* in South Africa. They looked at changes in density of these three hormones in one year. The highest amount of abscisic acid was found in both algae in September, and cytokinin was found in *Ulva* in July and in *Dictyota* in September.

Benitez-García *et al.* (2020) identified two gibberellins (GA_1 and GA_4) and abscisic acid (ABA) in *Padina durvillei* and *Ulva lactuca.* They found that the amount of GA_4 was significantly higher in *P. durvillaei* and ABA was greater in *U. lactuca*.

Research on seaweed costituents would help produce liquid bio-fertilizers for the purpose of soil conditioning, since seaweeds can contain a wide variety of phytohormones in significant quantities. Therefore, it is important to continue seaweed studies to reveal new algae species particularly rich in phytohormones (Yalçın *et al.*, 2020). Due to high density and diversity of brown and red algae in southern coasts of Iran, there is a potential to use them to extract growth regulators in order to increase growth of crops and orchards from algae products (Tavalabi-Dezfuli *et al.*, 2016). *Sargassum* and *Gracilaria* are among the most abundant macroalgae on northern shores of Persian Gulf, which are ecologically very important and economically viable (Sohrabipour and Rabii, 1999).

Existence of growth regulators and extraction of these hormones from different species of macroalgae is studied in Iran. Therefore, the objective of the present study was identification of plant growth regulators of extracts of the macroalgae *Sargassum muticum* and *Gracilaria corticata*, and evaluate use of these extracts as potential biofertilizer. Determination of growth regulators in plant tissues is quite difficult due to their trace quantities in the range of 10^{-10}–10^{-6} mol kg-1 and presence of accompanying compounds showing interferent effect as well (Yalçın *et al.*, 2020), so a new protocol was introduced. This research can be considered as a basis for more extensive research in the field of extracting various growth-regulating hormones from macroalgae in Iran.

Materials and methods

Sampling

Sargassum muticum and *Gracilaria corticata* were collected bimonthly at low tides from shores of Bushehr, Persian Gulf (50˚ 48' 53" N, 28˚ 54 ' 41" E) between January 2016 and November 2016. Water temperature, salinity, oxygen levels, and water pH, were measured at sampling site by thermometer, refractometer, oximeters, and pH determinants, respectively (APHA, 2005). For assessing seasonal variability of biomass and extracting phytohormones, both species were sampled three times by using quadrates of 50×50 cm (0.25 m^2) (Gharanjik and Rohani Ghadikolaei, 2009; Guiry and Guiry, 2014). After washing with distilled water and separation of epiphytes from thalli in sterile and acid-washed containers, *Sargassum muticum* and *Gracilaria corticata* samples were weighed (Yalçın *et al.*, 2020) and transferred to Razi Laboratory Complex of Science and Research Branch of Islamic Azad University in Tehran for extraction of ABA and GA_3.

Extraction of *ABA and* GA_3

There are some literature about extraction of phytohormones from seaweeds but for lack of available facilities and devices and due to problems in using existing protocols, we developed a new method for extraction of these phytohormones. Specifically, 10 g of samples poured into 60 ml of solution [methanol-chloroform ammonium hydroxide (2N)].

In order to dissolve the hormones well, samples were homogenised at 0°C temperature and dark. The homogenate was filtered through Whatman No.1 paper, and transferred onto a separatory funnel. Water was distilled twice and added while stirred vigorously and the surface phase (chloroform) was discarded. The lower phase (water-methanol) was collected and the remaining chloroform and methanol was evaporated by rotation in evaporator rotary (45°C), then its volume reached 35 ml with addition of water.

HCl (1N) was added to aqueous phase and pH decreased to 2.5 before transferring to a separatory funnel. Then 15 ml of ethyl acetate was added to the funnel and the upper phase was separated. Addition ethyl acetate was repeated twice and the upper phase of three replicates was collected. This solution contained GA_3, free ABA (Ergün *et al.*, 2002; Dobrev *et al.*, 2005; Wally *et al.*, 2012; Großkinsky *et al.*, 2014).

Analysis of plant growth regulatory hormones

The samples passed through a 45% poly Tetrafluoroethylene filter and then injected into the HPLC column. The solution components obtained by HPLC were separated using a C_{18} column, UV detector with 0.7 ml/min flow rate, and 0.2% acetic acid solvent and 95% methanol at 40°C (Hau *et al.*, 2000).

Statistical analysis

The effect of time on environmental factors (temperature, salinity, dissolved oxygen and pH) and on *Sragassum muticum* and *Gracilaria corticata* biomass and phytohormones were analysed using factorial analysis of variance (ANOVA) followed by Chi-squared test to determine samples' main effects.

For comparison of association between the environmental factors, abscisic acid, gibberellin and biomass, Pearson correlation coefficient was determined using SPSS Software version 21.0 (Khatami, 2003).

Results

Environmental factors

Results on the environmental factors are presented in Table 1. Temperature and dissolved oxygen were positively correlated with salinity while there was inverse linear relationship between DO and pH (p<0.05). there was significant and direct linear relationship between DO and salinity (p<0.05). Findings showed a strong but inverse linear relationship between salinity and pH.

Table 1: Average results of environmental factors in one year (± standard error; n=6).

Month	Temperature (ºC)	Salinity (ppt)	DO	pH
January 2016	28.0±0.52	44.33±1.01	2.16±0.09	7.12±0.24
March 2016	28.0±1.11	47.00±1.22	2.36±0.17	7.50±1.31
May 2016	32.0±1.16	44.66±1.35	2.22±0.12	7.51±0.10
July 2016	38.0±2.11	48.33±1.22	2.27±0.09	7.91±1.43
September 2016	35.0±1.84	40.33±0.89	2.80±0.35	7.16±0.36
November 2016	33.0±1.77	42.00±0.97	2.42±0.22	7.48±0.73
Average in 6 month	32.3±1.21	44.44±0/77	2.37±0.19	7.45±0.29
Max	38.0±2.11	48.33±1.22	2.80±0.35	7.91±1.43
Min	28.0±0.52	40.33±0.89	2.16±0.09	7.12±0.24

Temperature

Results of annual measurement of temperature in coastal waters of Bushehr region are shown in Figure 1. Average measured temperature during six months was highest in July with 38°C and the lowest in January and March with 28°C. The trend of monthly changes showed that temperature started to rise in January and reached its highest level in July. Temperature was almost the same in spring and fall. Results of Chi-square test and ANOVA showed significant difference among studied months (p<0.05; n=6).

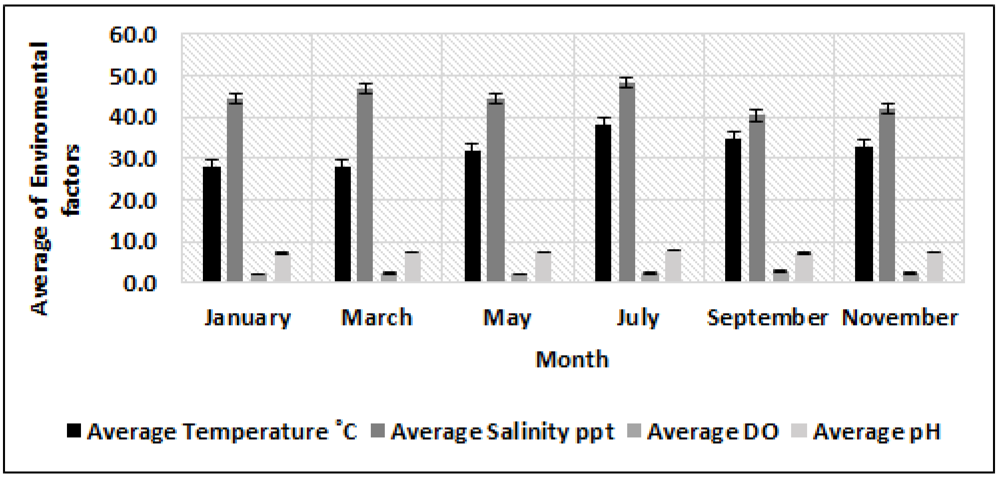

Figure 1: Comparison of environmental factors during one year (6 consecutive months) from Jan 2016 to Nov 2016. Error bars show standard error.

Salinity

Changes in salinity of seawater are shown in Figure 1. Average salinity during the six months of study reached a maximum of 48.33 ppt in July, and in September it reached its lowest level of 40.33 ppt. Average salinity was 44.44 ppt during a year. The trend of change during the six months of the study showed that salinity rate would peak in early summer. During fall, a decrease in salinity was observed, and gradually the trend of increasing salinity resumed in March. One-way ANOVA, as well as Chi-square test, showed significant difference among months (p<0.05; n=6).

pH

Results of annual measurement (six consecutive months) of pH in coastal waters of the study area are shown in Figure 2. During the study period, average pH reached a maximum of 7.91 in July and a minimum of 7.12 in January. The trend of pH changes over one year showed that pH was almost constant. Although it was observed that the average pH had the lowest amount in winter and the highest amount in summer, there was no significant difference between July and January in terms of average pH decline.

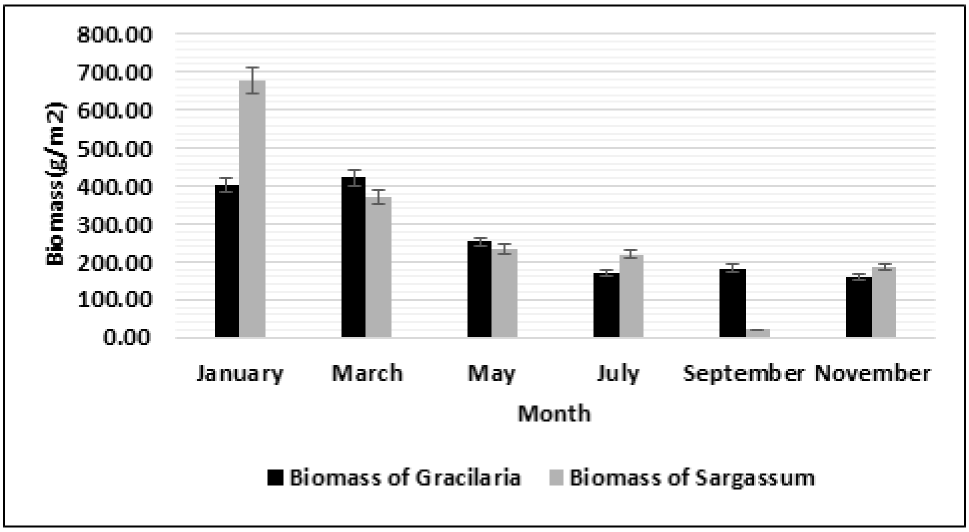

Figure 2: Comparison of average biomass of *Gracilaria* and *Sargassum* (g/m^2). Error bars show standard error.

Dissolved oxygen (DO)

Results of measuring oxygen levels in coastal waters of the study area over six months are shown in Figure 1. The highest average water-soluble oxygen was 2.80 in September and the lowest was 2.16 in January. A constant rend of changes in oxygen level was not observed and a relatively significant difference was observed regarding oxygen level among months.

Results of Sargassum muticum and Gracilaria corticata biomass from January 2016 to November 2016

As can be seen in Figure 2, the highest mean biomass of *Sargassum* (679 g/m^2) was observed in January 2016 ,while the lowest mean (20.66 g/m^2) was in September 2016 during one year of sampling. There was significant difference among bimass of *Sargassum* during six months of sampling (p<0.05; n=6). The highest mean biomass of *Gracilaria* (423.33 g/m^2) was related to March 2016, and the lowest mean (158 g/m^2) was in November 2016. There was significant difference among biomass of *Gracilaria* during six months of sampling (p<0.05; n=6).

Results of extracting regulating plant growth hormones from Sargassum muticum

Chemical structure of the phytohormones is shown in Figure 3.

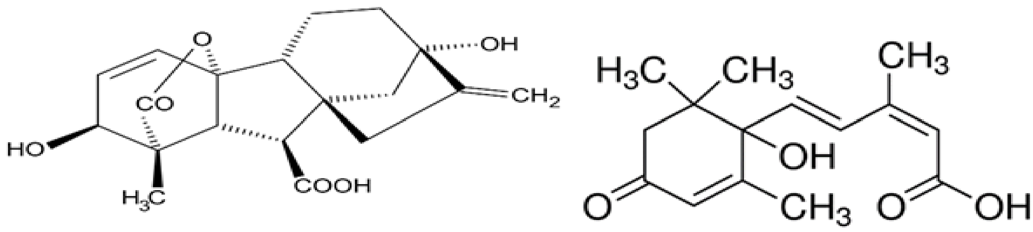

Figure 3: Chemical structure of gibberellic acid (GA_3) and abscisic acid (ABA).

Abscisic acid (ABA)

The results of extracting abscisic acid showed that the lowest amount of abscisic acid extraction from *Sargassum* was in July and May, 0%, and the highest rate of extraction was 20.667% (equivalent to 2.0667% of 1g of *Sargassum*) in November (Fig. 4). Chi-squared parent test and one-way ANOVA showed significant difference among 6 months of sampling during one year (p<0.05; n=6).

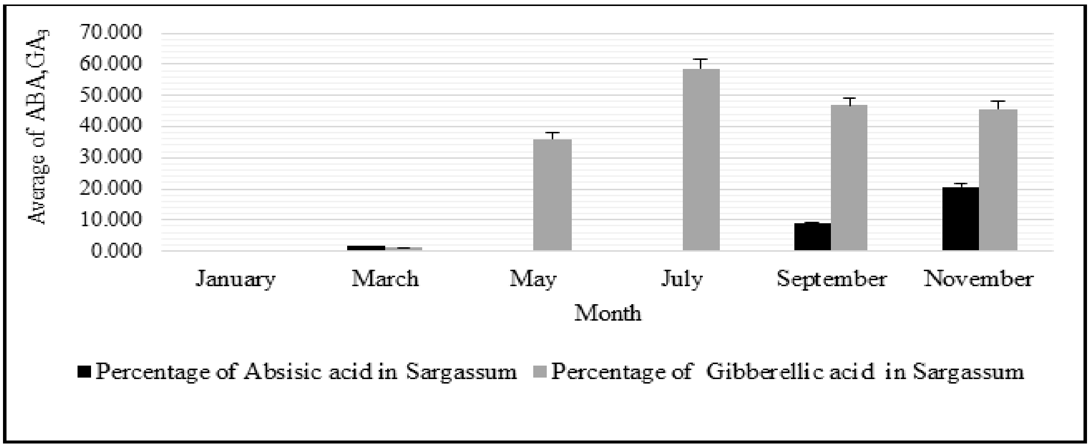

Figure 4: Average percentage of abscisic acid (ABA) and gibberellic acid (GA_3) extracted from *Sargassum muticum* from January 2016 to November 2016. Error bars show standard error.

Gibberellic acid (GA_3)

Results of gibberellic acid extraction from *Sargassum* algae are shown in Figure 4. The highest amount of gibberellic acid extracted from *Sargassum* was 58.561% (equivalent to 5. 8561% of 1g of *Sargassum*) in July 2016 and the lowest was obtained in January 2016 (0%). Chi-square test and one-way ANOVA showed significant difference among gibberellic acid in 6-month sampling period during one year (p<0.05; n=6).

Results of extracting hormone-regulating plant growth from Gracilaria corticata

Abscisic acid (ABA)

As can be seen in Figure 5, results of extracting abscisic acid showed that the lowest amount of abscisic acid of *Gracilaria corticata* was 0% and the highest amount was 66.20% in November 2016. Chi-squared parent test and one-way ANOVA showed significant difference among the 6-month sampling period over a year (p<0.05; n=6).

Gibberellic acid (GA_3)

The results of the present study showed that the highest GA_3 extracted from *Gracilaria corticata* was 84.467% in May 2016, and the lowest amount was 8.907% in November 2016 (Fig. 5). Chi-squared parent test and one-way ANOVA showed significant difference among the 6-month sampling period over a year (p<0.05; n=6). There was correlation between plant growth regulators such as *Sargassum* and *Gracilaria* algae and environmental factors.

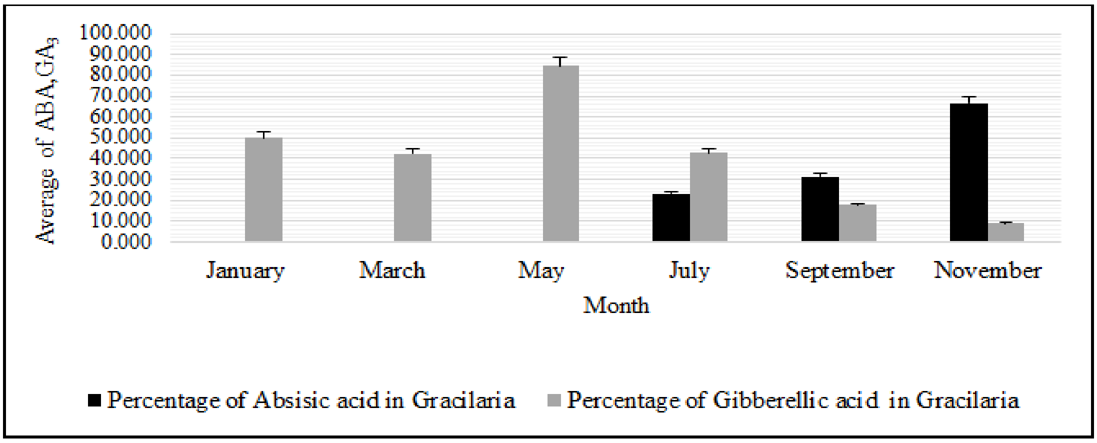

Figure 5: Average percentage of abscisic acid (ABA) and gibberellic acid (GA_3) extracted from *Gracilaria corticata* from January 2016 to November 2016. Error bars show standard error.

Discussion

This study aimed to investigate the effect of environmental factors on amounts of two plant growth regulators (ABA, GA_3) from *Gracilaria corticata* and *Sargassum muticum* from January 2016 to November 2016 (during one year).

Based on one-way ANOVA and Chi-squared test, mean squares of environmental factors (salinity, temperature, DO, and pH) showed significant difference among months (p=0.001). Observations also showed that there was significant correlation between environmental factors. These results agreed with Shahidi (2007), Shapori (2007), Gharanjik *et al.* (2011), and Hayati (2009). All of them found significant correlation between temperature and salinity, and between oxygen, pH and salinity. Biomass of seaweeds largely depend on season and environmental factors. This study showed maximum biomass of *Sargassum* in January (679 g/m^2) and minimum in September (20/667 g/m^2).

This is in agreement with Maureen *et al.* (2017) findings that development and growth period of *S. muticum* occured during winter and spring, and in summer the thalli begin to experience a deterioration indicated loss of buoyancy due to detachment of air bladder. A study on the effect of temperature on seasonality of *Sargassum* in Japan showed that sub-tropical *Sargassum* grow well at temperatures in range of 16–21. This temperature range coincides with seawater temperature in winter in southern Japan Sea (Nagai *et al.*, 2011). Shahidi (2007) and Gharanjik *et al.* (2011) reported increase in biomass from January to November from 197 to 650 and 200 to 860 g/m^2, respectively.

In Morocco growth of *Gracilaria multipartita* was maximum in spring and autumn, and the seaweed partially decayed after its maximum fertility was reached in June and October (Givernaud *et al.*, 1999). Seaweeds of *Gracilaria* sp.(chorda type), which grow along the coast of Uranouchi Inlet in Tosa Bay, southern Japan, showed highest biomass in summer and spring but gradually decreased in autumn and winter (Chirapart and Ohno, 1993). Subba Rangaiah (1983) found peak of biomass of *Gracilaria corticata* to be in January. In this study biomass of *Gracilaria corticata* was maximum in January and May with temperature 28°C. It is obvious that this species could not tolerate higher temperature degrees in summer. There was no correlation between temperature and biomass of *Sargassum* and *Gracilaria* but had positive correlation with salinity. The range of tolerance to salinity in seaweed species can be extremely variable and it is one of important factors influencing its growth. In study of Shahidi (2007), these factors mentioned as limiting factors for growth and reduction of biomass.

Coastal marine macroalgae assemblages are usually impacted by variation of salinity as a result of rainfall and runoff from the inland areas (Thambiraj *et al.*, 2012). Thus, most research into the effects of salinity on *Sargassum* growth revealed that this is a genus that can grow in a wide range of salinities and has a broad tolerance. Composition of phytohormones and their distribution in seaweeds are correlated with the algae species, seasonal changes and fluctuations, location from which they are collected, and growth phase of the algae (Yalçın *et al.*, 2020). Presence of ABA in seaweeds is reported in two brown algae, *Ascophyllum nodosum* and *Laminaria digitata* (Khan *et al.*, 2009). Gupta *et al.* (2011) determined plant growth regulators, including gibberellic acid (GA_3) and abscisic acid (ABA), in six species of green seaweeds (mostly *Monostroma* and *Ulva* species) from coasts of India. ABA was in range of 11.35–68.28 nmol g^{-1} fw.

Yalçın *et al.* (2020) reported presence of gibberellin in brown alga *Treptacantha barbata* and red alga *Polysiphonia scopulorum* and ABA in *Treptacantha barbata, Colpomenia* (brown algae), *Halopithys incurva, Gracilaria bursa-pastoris, Ellisolandia elongata* (red algae), *Penicillus* *capitatus* and *Flabellia petiolata* (green algae). The content of GA_3 had greater level than that of ABA in all of specimens. Brown algae is identified as the main source of plant growth regularors because of high content of active compounds and high availability throughout the year. In this study, the highest amount of GA_3 and ABA were found in *Gracilaria corticata* (84.467% and 66.20%, respectively). Mikami *et al.* (2016) reported two red seaweeds, *Pyropia yezoensis* and *Bangia fuscopurpurea* to contain ABA 1.2 and 1.3 ng g^{-1}dw, respectively. In eleven red seaweeds from Brazil coasts, including *Chondracanthus teedei* ABA was detected in range of 0.019–8.9 and 0.01–0.058 nmol g^{-1}dw, respectively. These results confirmed that phytohormones like ABA were common components in red seaweeds. This was the first report of occurrence of ABA in Rhodophyta (Yokoya *et al.*, 2010).

According to results of abscisic acid (ABA) and gibberellic acid (GA_3), this study showed that amount of ABA in *Gracilaria corticata* increased from January to May and reached its maximum in May during one year. ABA is a growth inhibitor and makes the plant to go dormant and GA_3 is a growth stimulant and increases length of the yarn (Benková, 2016).

Abscisic acid (ABA) has an important signaling role in enhancing plant tolerance to environmental stress (Guajardo *et al.*, 2016). The hormone ABA regulates oxidative stress state under desiccation in seaweed species; an environmental condition generated during daily tidal changes (Liu *et al.*, 2018). Thus in this study high temperature in July, September and November (38, 35 and 33°C) followed by desiccation stress in intertidal zone caused increase in amount of abscisic acid. Accordingly, the amount of biomass in *Gracilaria* had negative correlation with abscisic acid . This is due to inhibitory effect of abscisic acid on growth. Amount of GA_3 increased and reached its maximum in November. There was no significant relation between gibberrellic acid and biomass of *Gracilaria*.

Concerning the brown alga *Sargassum muticum*, the amount of ABA increased from March to July and reached its maximum in July. The amount decreased since September with presence of GA_3. GA_3 levels peaked in November. Although *Sargassum* biomass was declining from January to September due to limiting environmental factors, the *Sargassum* biomass declined unexpectedly with the onset of warm season and increase in GA_3. It has reached its minimum level in September. The biomass of *Sargassum* had negative correlation with abscisic acid. With the increase of abscisic acid, growth inhibitor hormone, amount of biomass decreased. Strike *et al.* (2009) extracted cytokinin, auxin, and abscisic acid from *Ulva* and *Dictyota* algae in South Africa and examined changes in density of these three hormones during one year. Auxin was highest in *Ulva* algae in March and in *Dictyota* in May; abscisic acid was highest in both algae in September and cytokinin was highest in

Ulva in July and in *Dictyota* in September. In the present study, abscisic acid was high in *Sargassum* in September too.

Findings of this research showed that the amount of algal biomass increased in winter. During spring and summer amount of biomass of both algae decreased and again increased in fall. Significant differences were observed among different months in terms of algae biomass during the 6-month sampling period ($p<0.05$; n=6).

Environmental factors such as temperature and salinity have an effect on biomass of *Sargassum mutium* and *Gracilaria corticata* in presence of growth inhibitor (ABA) and growth stimulant (GA_3). In general, with decrease of ABA hormone, GA_3 hormone increases, and vice versa. Given the exorbitant price of plant hormones, the need to use these hormones in algae liquid fertilizers, extensive use of algae extracts in agriculture and fisheries, and presence of many macroalgae on shores of Persian Gulf, this study can be considered as a useful source for extraction of a variety of hormones from these algae.

In this work, a new method was developed for extracting phytohormones composition of seaweeds and existance of hormones suggests that plant hormones play a role in regulating physiological processes in Rhodophyta and Phaeophyta.

Acknowledgment

The authors sincerely thank Mr. Reza Mohsenian, Head of Department of Basic Sciences Laboratories of Islamic Azad University, Tehran Science and Research Branch for their support and guidance, Mr. Amir Hossein Zardchashmi who helped financing the project and dear translator, Ms. Fatemeh Zardchashmi. We would also like to thank dear experts of Razi Laboratory of Islamic Azad University, Tehran Science and Research Branch for their cooperation and sincere assistance. Finally, we express our gratitude towards all those who helped us in this project.

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