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Analysis of bioactive compounds from *Gracilaria foliifera* based on lunar phases

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Gracilaria foliifera, sustainable renewable resources in the marine environment. *Gracilaria*, a genus of red algae, notable for its economic importance as an agarophyte. In the present study, experiments were performed to investigate the phytochemical constituents of *Gracilaria foliifera*. Samples were collected during three different lunar phases namely new moon, full moon and between days. The collected seaweeds were shade dried and extracted by ethyl acetate. The crude metabolites are subjected to phytochemical analysis, antioxidant activity, and qualitative analysis of the compounds by TLC. Further the crude extract was evaluated by GCMS. Among the different lunar phases, the presence of phytochemical compounds, antioxidants activity, is maximum during the full moon days which also showed appreciable amount than the samples collected during new moon phase and transition phase.

[**Keywords**: Bioactivity, Full moon, GC-MS analysis, *Gracilaria foliifera*, New moon]

Introduction

Seasonal variations are known to profoundly affect the growth of food crops, herbs, higher plants, marine algae and their metabolic activity. The intertidal zones and tropical waters of the oceans are home to more than 150,000 species of seaweed, making up more than 80 % of the world's plant and animal species. They are the main source of naturally occurring bioactive compounds. Seaweeds are shallow marine meadow plants that float and submerge. They have salt tolerance and may prevent desiccation by altering the osmolarity of their cytoplasm to match that of saltwater[1]. They don't have prominent stems, roots, or leaves, but they do have stipes, holdfasts, and blades that resemble leaves, stems, and roots in terrestrial plants. With the help of sunlight, seaweeds with photosynthetic pigments make food and oxygen from carbon dioxide and water[2].

Marine macroalgae are most wanted for both ecological and economical reasons in many parts of the world, particularly in Asian nations like China, Japan, and Korea. They are an excellent source of food that is low in calories and high in vitamins, minerals, proteins, polysaccharides, steroids, and dietary fibres. They were also being active ingredient in traditional treatments as far back as 3000 BC. Brown algae were used in the treatment of hyperthyroidism and other glandular diseases by the Chinese and the Japanese. Unsaturated fats also provide defense against microorganisms that affect the cardiovascular system[3].

Among the richest and most promising sources of bioactive primary and secondary metabolites, seaweeds are one of the major bioresource for carrying out research over the past three decades. These macroalgae produce a wide range of substances, including carotenoids, terpenoids, xanthophylls, chlorophyll, vitamins, saturated and polyunsaturated fatty acids, acetogenins, amino acids, antioxidants like polyphenols, halogenated substances, alkaloids, and polysaccharides like agar, carrageenan, alginate, proteoglycans, rhamnan sulphate, laminaran, galactosyl glycerol and fucoidan[4,5]. These substances applied as antifouling, allelopathic, antibacterial, herbivore deterrents, or ultraviolet-screening agents as a result of their diverse range of functions. They provide the pharmaceutical industry with medications to treat illnesses like cancer, AIDS, pain, inflammation, arthritis, and infections from bacteria, viruses, and fungi. At the

moment, algae account for around 9 % of marine medicinal chemicals[6]. In green, brown, and red algae, many bioactive substances having cytostatic, antihelmintic, antiviral, antifungal, and antibacterial properties have been found. Many regions of the world use red seaweeds of the species *Gracilaria* as fresh food[7].

In view of the diversified bioactivity offered by the seaweeds through varied compounds it produces, we tried to study the influence of lunar phases namely full moon and new moon on the production and activities of metabolites produced by the sea weeds. To the best of our knowledge, this is the first report on the effect of lunar phases on *Gracilaria foliifera* bioactive compounds.

Materials and Methods

Sample collection and preparation

The *Graciliaria foliifera* was collected from the rocky shore of Kovalam beach, Chennai, India (12°47' N; 80°15' E) during three different lunar phases *i.e*., during the full moon, new moon and in between the days. The collected samples were washed with tap water for several times and shade dried. Later on the sample was made into powder with help of mixer and stored for future use.

Solvent extraction

The powdered sample (5 g) was extracted with 50 ml of ethyl acetate and incubated overnight under shaking condition. This was followed by centrifugation at 3000 rpm for 10 min. The supernatant was collected and evaporated to obtain the crude sample which was resuspended in 1 ml of ethyl acetate.

Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methods. Alkaloids were detected by Mayer's test and Dragendroff's test. The occurance of yellow colour and red colour respectively indicates the presence of alkaloids. Carbohydrates were determined by Molisch's test and Benedict's Test. Glycosides were detected by modified Borntrager's Test. Saponins were detected by Foam Test. Phytosterols were identified by Salkowski's Test and Libermann Burchard's test. Phenols and tannins were determined by Ferric Chloride Test. Proteins and aminoacids were detected by Ninhydrin Test, Millon's test, Biuret test. Flavanoids, terpenoids and theobromine were also detected[8].

Quantitative estimation of phytochemicals

Proteins

The aromatic amino acids tyrosine and tryptophan present in the crude extract reacts with Folin's phenol reagent to give a blue colour complex. The colour intensity was measured calorimetrically at 620 nm. 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the working standard solution of protein is pipetted out into a clean dry test tube marked as S1 to S5, respectively. The obtained crude extract was taken as 0.5 and 1.0 ml into the test tube marked as T1 and T2 respectively. The final volume is made up to 3.0 ml with distilled water. 1.0 ml of distilled water served as blank. 4.5 ml of alkaline copper reagent was added to all the tubes. The contents were mixed well and kept it in a room temperature for 30 min. The intensity of colour developed is read colorimetrically at 620 nm. A standard graph was drawn from the obtained values. From the graph the amount of protein present in the given unknown solution was calculated[9].

Carbohydrate

The carbohydrate content was analyzed using Anthrone method[10]. For Anthrone reagent preparation, 100 mg of Anthrone was dissolved in 50 mL of ice-cold crude 96 % sulfuric acid. For total carbohydrate measurement the reaction mixture was boiled at 100 °C for 10 min and cooled at room temperature. Then the absorbance was determined at 630 nm wavelength.

Flavonoids

The aluminium chloride colorimetric assay was used to determine the total flavonoid concentration. In a 10 ml volumetric flask with 4 ml of distilled water, an aliquot (1 ml) of extracts and standard solutions of quercetin (20, 40, 60, 80, and 100 g/ml) was added. After five minutes, 0.3 ml of 10 % $AlCl_3$ and 0.30 ml of 5 % $NaNO_2$ was added to the flask. After waiting for five minutes, 2 ml of IM NaOH was added, and 10 ml of distilled water was added to make the volume. At 510 nm, the mixture's absorbance was measured in comparison to a blank. The amount of flavonoid in total was measured in mg of quercetin equivalents[11].

Phenols

The Folin-Ciocalteu test was used to calculate the total phenolic content. To 25 ml volumetric flask containing 9 ml of distilled water, an aliquot (1 ml) of the extract and a standard solution of gallic acid (100, 200, 300, 400, and 500 g/ml) were added. Distilled water was used as blank. The mixture was mixed with

1 ml of the Folin-Ciocalteu phenol reagent before shaking. 10 ml of 7 % Na_2CO_3 solution was added to the mixture after 5 minutes. UV-Visible spectrophotometer was used to measure the absorbance at 550 nm after 90 minutes at room temperature incubation. mg Gallic Acid Equivalents (GAE) were used to express the total phenolic content.

Thin layer chromatography

To qualitatively examine the components of the *Gracilaria foliifera* extract, a TLC standard plate measuring 2.5 x 7.5 cm was used. At the bottom of the plate, the sample extracts were dabbed and given time to dry. In the solvent tank with 1:1 n-hexane and ethyl acetate the plate was placed. Movement of bands was allowed to run for three-fourths of the distance after which it was dried. The spots were visualized by spraying reagent. This entails spraying with a spotting reagent and viewing the object under UV light. The RF value was determined using

RF = Distance travelled by the solute / Distance travelled by the solvent

Antioxidant activity

The free radical scavenging activity of seaweed extracts and of standard solution (ascorbic acid) was investigated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method as reported by Asadujjaman *et al*.[12] using Ascorbic acid as standard. Briefly, 0.1 mM of ethanolic DPPH radical solution was prepared in ethanol and initial absorbance was measured at 517 nm. An aliquot (1.0 ml) of each sample (with appropriate dilution 100 µg/ml) was added to 3.0 ml of ethanolic DPPH radical solution. Decrease in colour was measured after incubation in dark for 30 min.

% scavenging activity = $Abs_{control} - Abs_{sample}/Abs_{control} \times 100$

GC-MS analysis of the extract

The extract was analysed by GC-MS utilising a triple quadrupole mass spectrometer with fused silica capillary columns that were 30 mm long, 0.25 mm thick, and had a diameter of 30 mm. The column oven's temperature ranges from 70 °C to 300 °C, and kept at that level for 2 to 10 minutes. Helium flow rate was set to 1.5 ml/min, and the injection port temperature was guaranteed to be 240 °C. EI was the ionisation voltage (-70ev). The samples were separated into 10 separate injections. The 40–1000 (m/z) mass spectral scan range was chosen. Compounds that are present in the extracts are found by using NIST08s, WILEY8 and FAME MS data library and compared the spectra obtained using GC-MS-MS for all three samples.

Results and Discussion

The samples of *Gracilaria foliifera* at different lunar phases (Full Moon (FM), New Moon (NM) and between days or Transition Phase (TP)) were collected. At each phase, 3 samples were collected and extracted using ethyl acetate as solvent after which they were dried and the powder thus obtained was first analysed for the presence of various phytochemicals.

The *Gracilaria foliifera* was gathered for over a period of 4 months and the phytochemical analysis was carried out for the samples and the data is given in (Table 1).

The phytochemical analysis of the seaweed samples obtained during full moon showed the presence of alkaloids, carbohydrates, phytosterols,

Table 1 — Biologically active chemical compounds of *Gracilaria foliifera* ethyl acetate extract during different lunar phases

Peak	Compounds	RT	New moon (Area %)	Full moon (Area %)	Transition phase (Area %)
1	Hexadecanoic acid	19.537	47.7	61	52.327
2	Vaccenic acid	20.045	12.694	0.476	-
3	Arachidonic acid	22.464	4.182	1.431	-
4	Eicosapentaenoic acid	22.531	3.763	1.467	-
5	Cis-13- Octadecenoic acid	21.075	-	-	5.826
6	Octadecenoic acid	21.208	3.298	11.896	10.351
7	Tetradecanoic acid	17.227	3.231	3.183	-
8	Heptadecanoic acid	20.324	-	1.768	0.764
9	Dodecanoic acid	14.983	2.917	0.911	-
10	Pentadecanoic acid	18.244	2.609	1.558	0.302
11	Phytol	20.696	1.06	0.476	2.149
12	Palmitoleic acid	19.09	0.655	0.664	0.932
13	Benzofuranone	14.693	-	0.323	-
14	Ascorbic acid	18.846	-	-	0.226

proteins, flavonoids, terpenoids and threobromine. Glycosides, Tannins and saponins were absent in the samples. The presence of the phytochemicals was an immediate reaction indicating its presence in good quantities in the samples collected at different time intervals. Ghannadi *et al.*[13] reported that similar kind of phytochemicals are found in *Gracilaria salicornia* and *Gracilaria corticata*[3]. Similarly, the phytochemical analysis was carried out for NM-extract and this showed the presence of carbohydrates, alkaloids, phytosterols, proteins, flavonoids, terpenoids and threobromine; while Tannins, Glycosides and saponins were absent. Moreover, carbohydrates and theobromine were shown to be obtained as a slow reaction indicating their presence in low quantities. The presence of phytochemicals in the transition phases was also observed and it was noticed that carbohydrates and theobromine showed variation. In quantification of phytochemicals determination, full moon phase samples showed maximum content of carbohydrates (12.06 %) compared to new moon (9.266 %) and transition phase (5.33 %) samples. Similarly, percentage of proteins and flavonoids varied between (2.35 %, 2.25 %, 1.45 %) and (1.48 %, 1.40 %, 1.25 %) during full moon, new moon and transition phase, respectively. New moon phase collected samples contain higher phenol content (2.16 %), compared to full moon (1.39 %), and transition phase (1.1 %).

Thin Layer Chromatography (TLC) analysis

TLC analysis of three lunar phases samples revealed the presence of bands in the full moon extract with retention time (0.87, 0.64, and 0.86), new moon extracts (0.80. 0.92, 0.63) which indicates the presence of different classes of compounds. Transition phase extracts also revealed the presence of 2 phytochemical bands with retention time 0.69 & 0.72, 0.78 & 0.82, 0.80 & 0.82 thereby clearly showing a variation in the nature of compounds and the influence of the lunar phases on the growth and production of phytochemicals. Though the results clearly infers provides basic information on the possible compounds, a detailed instrumental analysis has to be performed. The chemical composition of *G. foliifera* was collected on lunar phases. The maximum phytochemical contents were found in full moon samples followed by new moon and transition phase. Previous study confirmed that *Gracilaria* contained a high total phenolic content, vitamin E, vitamin C and the antioxidant capacity[14].

Antioxidant activity of the NM, FM and TP extracts

The antioxidant activity of the extracts of different lunar phases was evaluated using ascorbic acid as standard. From the results it was observed that there is an increase in the DPPH scavenging activity with the increase in the concentration of the extract. The FM and NM extracts showed activity on par with the standard used but compared to NM extract, FM - extract showed appreciable amount of activity. This clearly suggests that the influence of lunar phase on the potential of metabolites.

While the activity of full moon phase and new moon seaweed extract were little higher than the transition phase, the extracts obtained from the full moon phase showed increased DPPH scavenging activity compared to that of the control. The antioxidant activity of full moon phase was higher and linear up to 49.98 µg with reference to the inhibition rate. This indicates that the extracts are full moon phase had a reservoir of vibrant antioxidant compounds.

Though the extracts from the lunar phases showed promising antioxidant activity, the extracts from the transition phase showed moderate activity which was less than the control. These results clearly suggest that there is a tremendous influence of the lunar phases on the production of secondary metabolites that showed significant antioxidant activity.

The results obtained in the present investigation is well supported by a study conducted by Chejara *et al.*[15] where the antioxidant potential of two seaweeds *Gracilaria pudumadamensis* and *Dictyopteris australis* were reported upto 82 % in DPPH activity. This activity may be due to the presence of phenols, steroids and terpenoids. Additionally, *Halimeda tuna*, *Turbinaria conoides* and *Gracilaria foliifera* were found to show good antioxidant activity having higher phenol content as reported by Devi *et al*.[16]. Also, another report by Gouda *et al.*[17] demonstrated a strong antioxidant activity by *Gracilaria verrucosa* in comparison to the standard commercial Butylated hydroxyl toluene. Seaweeds from Bangladesh were also found to possess high phenol content with high antioxidant activity[18]. Another species *Gracilaria edulis* also proved to be a potential source of antioxidant compounds with significant activity based on Scavenging activity of ABTS, superoxide radical, hydrogen peroxide and DPPH[19]. Research was also carried out in development of a formulation by encapsulating the antimicrobial and antioxidant bioactive compounds into using beads against

A. salmonicida infection in *O. mossambicus*[20]. Also the anticancer potential of *Gracilaria foliifera* have been studied and reports suggested that they can be novel source for anticancer compounds[21,22]. Though reports are existing on the antioxidant potential of *Gracilaria foliifera*, this work attempts to find the effect of lunar phases on the bioactive potential of the sea weed and the results clearly indicate there is an effect of lunar phases on the seaweed and thereby its ability to produce metabolites of medicinal importance.

GC-MS analysis of the extract

The extracts' GC-MS analysis revealed the presence of the chemicals in all phases of the moon, including new moon (Table 1; Fig. 1), full moon (Table 1; Fig. 2) and transitional phase (Table 1; Fig. 3).

The antioxidant activity of these seaweeds would increase their usefulness in the human diet as food and pharmaceutical supplements because marine algae are a rich source of dietary fibre, minerals, proteins, and vitamins. There is a lot of room for more research in the area of drug creation. Several reports are there to determine the numerous types of microalgae that produce important substances including tetradecanoic acid, hexadecanoic acids, octodecanoid acid methyl esters, etc.[23]. High levels of

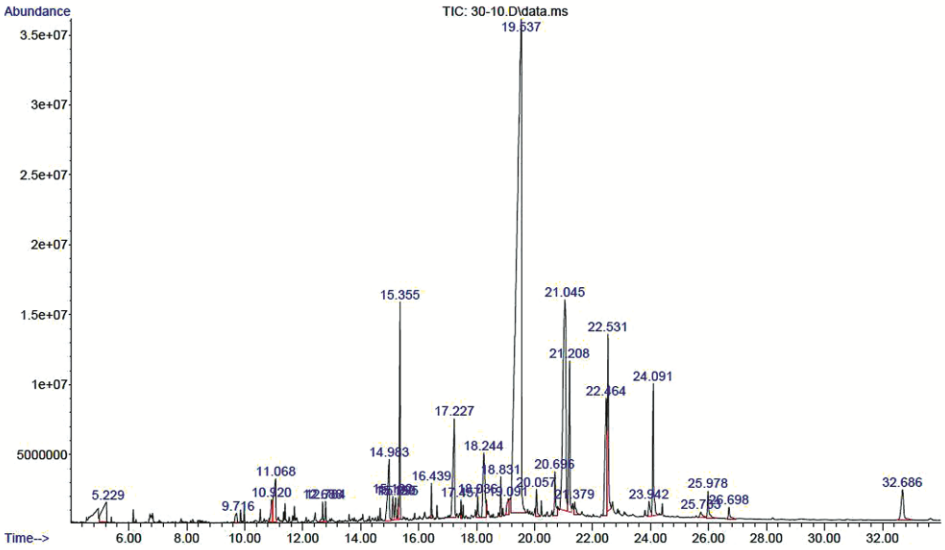

Fig. 1 — Evaluation of bioactive compounds by GC-MS analysis of *Gracilaria foliifera* ethyl acetate during new moon phase

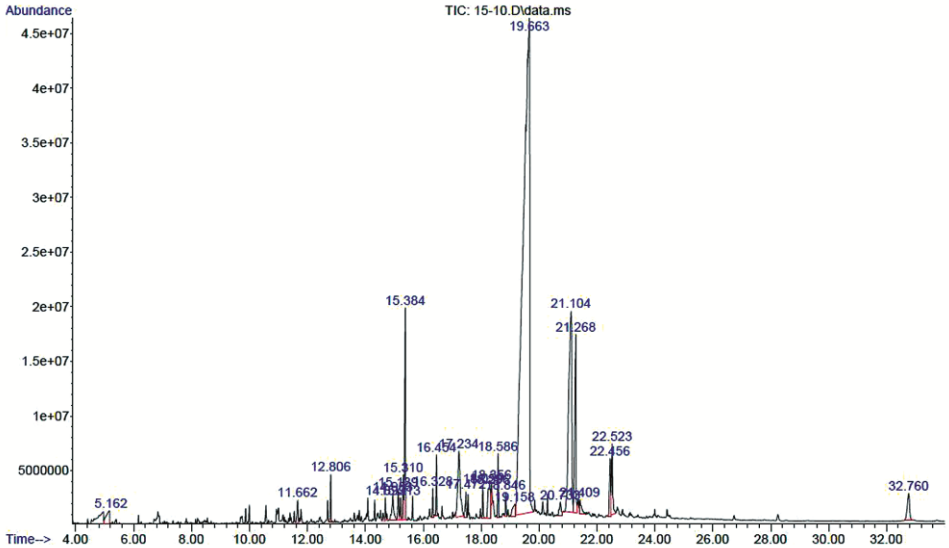

Fig. 2 — Evaluation of bioactive compounds by GC-MS analysis of *Gracilaria foliifera* ethyl acetate during full moon phase

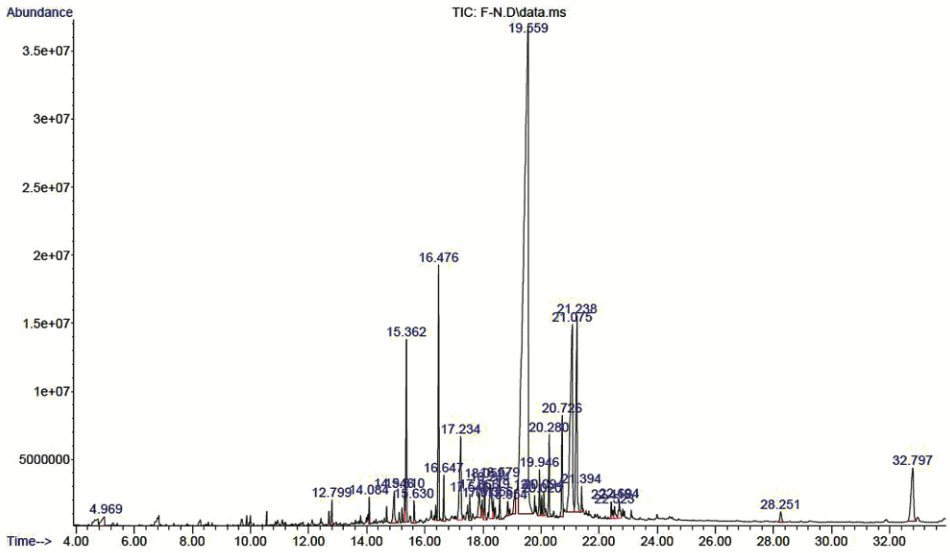

Fig. 3 — Evaluation of bioactive compounds by GC-MS analysis of *Gracilaria foliifera* ethyl acetate extract during transition phase

fatty acids found in marine algae indicate utilization for nutrient supplement and possible bioactivity[24]. The new moon sample contains a variety of substances, including hexadecanoic acid (47.10 %), vaccenic acid (12.69 %), octadecanoic acid (3.29 %), arachidonic acid (4.18 %), eicosapentaenoic acid (3.76 %), phytol (1.06 %), and palmitoleic acid (0.65 %). Due to the fact that some fatty acids have been demonstrated to have antibacterial properties, the lipids and fatty acids from marine algae are crucial as precursors for many other bioactive secondary metabolites[25,26]. Similarly in full moon samples, hexadecanoic acid (48.25 %), vaccenic acid (0.476 %), octodecanic acid (9.54 %), palmitoleic acid (0.664 %), phytol (0.476 %), eicosapentaenoic acid (1.46 %), arachidonic acid (1.43 %) were found; whereas the transition phase samples contained hexadecanoic acid (55.614 %), octadecanoic acid (11.896 %), phytol (10.787 %), Cis-13- octadecenoic acid (11.708 %), palmitoleic acid (0.798 %), arachidonic acid (1.431 %) and ascorbic acid (0.22 %). In these three phase samples hexadecanoic acid and archidonic acid were found to be in maximum concentration. But higher content of phytol was observed in transition phase when compared with new moon and full moon sample. Similarly maximum amount of vaccenic acid was present during new moon followed by full moon sample but absent in transition phase. In new moon sample maximum amount of arachidonic acid and eicosapentaenoic acid could be observed than other two-phase samples. Palmitoleic acid present in all extracts but transition phase contains relatively near high. Cis-13-octadecenoic acid and ascorbic acid could observe in transition phase but not found in new moon and full moon samples. This comparative analysis among samples obtained from transition phase, full moon and new moon sample revealed that the presence of varied compounds in higher concentrations was noticed in full moon and new moon phase compared to transition phase suggesting the possible influence of lunar phases maximum production of metabolites. This study demonstrates that lunar phases influenced the metabolic activity of *Gracilaria foliifera* characterized by the identification of varied compounds.

As per the literature survey conducted, not much of research has been conducted on the effect of lunar phases in *Gracilaria foliifera*. Earlier studies were conducted to understand effect of lunar phases on the egg laying capacity was observed in *Sparus aurata* which showed significant increase in the quantity of eggs in full moon condition compared to that of new moon which could be because of the tidal action during these phases[27].

A study was conducted by Bokhtiar *et al.*[28] to assess the effect of different factors like lunar cycle, rope type, harvesting interval, and seeding gap on *G. tenuistipitata* in coast of Cox's Bazar for a period of sixty days. It was observed that moon cycle influenced the seeding and harvesting thereby suggesting the influence of lunar cylces on growth of the seaweed.

Observations over a period of 18 years by Burnaford *et al.*[29] have revealed that climatic

conditions and emersion time significantly influences the ecosystem and therefore the existence of these vulnerable species in that condition. A 14 year field survey has showed variation in kelp canopy for *Saccharina sessilis* which was based on annual emersion time. Lab studies have shown that brief exposure to realistic low-tide conditions diminished physiological functioning and caused considerable biomass loss while *Katharina tunicate* had an opposite effect with increased canopy cover. Another study supported the fact that lunar phases affect the secondary metabolite production and antibacterial activity against Staphylococci using the extracts of *U. lactuca*[30].

This states that the natural climatic and celestial cycles have a profound effect on the flora and fauna in that ecosystem in terms of growth and physiological functioning.

Conclusion

Marine flora and fauna remain an unexplored source for a number of biological compounds that plays vital role in medicine and industry. This is due to the impact of environmental conditions and nature of ecosystem they survive in for normal physiological conditions. In the present study, a marine seaweed *Gracilaria foliifera* was obtained under various lunar phases including full moon, new moon and transition phases to understand the effect of lunar phases on the production of phytochemicals. Quantitative analysis of new moon samples showed higher phenol content along with greater antioxidants activity than other phase extracts. In TLC analysis, the transition phase sample showed the presence of two bands indicating less number of bioactive compounds compared to other phases. The GC-MS results of new moon and full moon samples showed the presence of various fatty acids and phenolic compounds compared to transition phase extracts. Therefore, this study demonstrates that samples collected during full moon and new moon showed higher level of metabolically active compounds when compared to transition phase samples. More supportive research has to be carried out in future so as to reap the benefits of marine flora in terms of biologically active compounds.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Statement

This work was not published in any other journal and no endangered species were being used in this study.

Author Contributions

All the authors have contributed to the work equally.

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