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Assessment Of Red Algae as A Source of Industrially Relevant Biomolecules: Biochemical Composition and Metabolic Fingerprinting

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Abstract
In order to get fresh red seaweed specimens for this investigation, many coastal locations of Gujarat were examined. In order to achieve optimal sampling efficiency, the collection was carried out during low tide and by using weather forecasts. The metabolic profile of Rhodophyta was characterised by subsequent metabolomic and biochemical investigations. Rhodophyta, or red algae, are a promising source of bioactive substances that have potential use in industry. In this work, we used gas chromatography-mass spectrometry (GC-MS) profiling in combination with a biochemical and metabolomic investigation of Rhodophyta, and also with a particular emphasis on lipids, carbohydrates, and chlorophyll. While GC-MS analysis identified the presence of a variety of bioactive chemicals, such as amino acids, hydrocarbons, organic acids, fatty acids, sugars, and other organic metabolites, Notably, Rhodophyta's potential as a natural addition in cosmetic compositions is highlighted by the presence of R-phycoerythrin, a desirable red pigment. Priorly, the results validated red algae's antioxidant qualities and highlight their use in practical applications. This work highlights the potential of Rhodophyta as a sustainable supply that offers fresh insights into the biochemical richness of this organism.

Keywords: Rhodophyta, Biochemical analysis, GC-MS profiling, Metabolite profiling, Natural pigments

Introduction:
97% of the Earth's water is marine and contains organisms such as fish, marine mammals, algae, coral, bacteria, octopuses, and crabs, with algae comprising 90% of the total marine flora, it is evident that marine algae hold significant potential for the extraction of commercially valuable compounds and their future applications in various fields. Kumari et al., (2022), López et al., (2021), Duarte et al., (2005). Among all the seaweeds such as green seaweed, brown seaweed and red seaweed; red seaweed consisting of more than 7,000 species, scientifically classified as Rhodophyta, represents a diverse group of marine algae distinguished by their intense red colouration, which is among their most recognisable features. The red pigment phycoerythrin not only imparts an appealing colour to red seaweed but also plays essential biological roles that facilitate the seaweed's survival, growth, and adaptation. Prasanna et al., (2007), Peñalver et al., (2020).
Because it contains essential vitamins, minerals, and antioxidants that promote a variety of metabolic processes and overall welfare, seaweed has a complex and significant impact on human health. Positive benefits on digestive, cardiovascular, and immune system health may also result. Sonani et al., (2016), Reddy et al., (2010). One of the prominent pigments that red algae make is R-phycoerythrin (R-PE), a water-soluble phycobiliprotein that gives these organisms their distinctive red hue. R-PE's antioxidant qualities and possible uses as a natural colourant and bioactive component in cosmetics have been the subject of much research. Its significance in cosmeceutical formulations is further shown by its capacity to stimulate collagen formation and demonstrate anti-allergic properties. Lee et al., (2021).
Red algae are distinguished by their significant levels of sulfated polysaccharides, phycobiliproteins, essential fatty acids, and a variety of secondary metabolites. These substances are useful for the pharmaceutical, nutraceutical, and cosmeceutical sectors because they have a variety of biological actions, such as anti-inflammatory, anti-cancer, antioxidant, and antibacterial qualities. Carpena et al., (2023).
Red seaweed metabolic profiling sheds light on the biochemical makeup and possible use of these organisms. One essential analytical method for locating and measuring volatile and semi-volatile substances in intricate biological matrices is gas chromatography-mass spectrometry (GC-MS). A variety of bioactive components found in red algae, such as amino acids, fatty acids, sugars, and organic acids, have been effectively identified by prior GC-MS research, demonstrating their diverse range of uses. Halket et al., (2005), Chong and Xia, (2018), Chong et al., (2018), Chong et al., (2019).
There is a good chance to investigate the metabolomic profiles of native red seaweed species

in Gujarat's coastal areas, which are home to a variety of marine biodiversity. In addition to advancing knowledge of the local marine flora, these studies open the door for the sustainable use of these resources across a range of businesses. Clarifying the metabolic and biochemical makeup of red seaweeds gathered from Gujarat's coastline is the goal of this study. Our objective is to discover the potential of these marine species as sustainable sources of bioactive chemicals for commercial uses by combining GC-MS profiling with focused investigation on content present in interested sample of seaweed. Paidi et al., (2017).

Materials and Methods:

Using the cleaning solution, every piece of glassware, including conical flasks, beakers, test tubes, pipettes, glass vials, Petri plates, etc., was cleaned before use.

Red seaweeds, particularly those belonging to the phylum Rhodophyta, have garnered a lot of attention due to their varied biochemical composition and potential health benefits. The morphology of the red algae sample was used to identify it. Using a little knife and the procedure outlined in the manuscript, fresh red seaweeds were harvested from the previously indicated collecting places by quickly cutting close hold without disturbing the algal bloom.

To remove any possible debris, sand, or epiphytes, the obtained algal sample was meticulously cleansed with seawater at the algal sample collection location. Individually, the gathered seaweeds were stored in sterile plastic bottles and polythene bags before being transported to the lab for pigment extraction. After collecting algae and washing them three times with tap water and once with distilled water, the seaweeds were shade-dried on filter paper to remove any remaining water. They were then kept in a deep freezer at -20°C and lyophilized half of the biomass to use for additional research. biochemical and metabolomic investigation of the obtained algal sample here further demonstrated.

Extraction and Estimation of chlorophyll by using methanol:

The algal biomass was used for the extraction and quantification of chlorophyll by using the methanol technique. Fill glass tubes with the ground seaweed paste. Fill the tubes with methanol to immerse the seaweed. To enable pigment extraction, place the tubes in a dark area and incubate them for 12 hours at 4°C or in a refrigerator. To separate the pigment extract and move the supernatant to sterile quartz cuvettes, centrifuge the tubes for 5 to 10 minutes at 3,000 rpm after incubation. Using a UV-visible spectrophotometer, the optical density of the supernatant containing the extracted pigments was measured at 663 and 688 nm for Chl. a and Chl. d, respectively. The protocol has been carried out and replicated three times. The means ± SD were used to express the data that we can see here in figure 1.

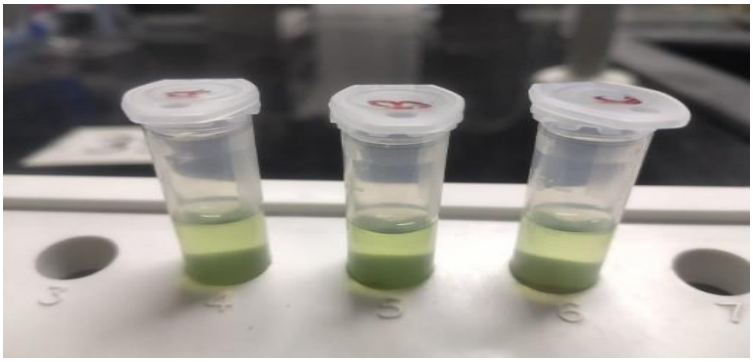

Figure 1: Chlorophyll extracted from 3 different algal samples (result ma)

Extracting and estimating total carbohydrates by phenol-sulfuric acid method:

A 100 mg sample of lyophilised seaweed has been used to extract the carbohydrates. To the seaweed sample, add 1 millilitre of distilled water. To extract the carbs, vortex the mixture well and heat it in a water bath at 80°C for approximately half an hour. Let the mixture cool until it reaches room temperature. Take 100 µL of the seaweed extract solution. Add 100 µL of the phenol reagent, or an equivalent volume, to the sample. Stir everything together. To create a layer underneath the phenol-sample combination, carefully pour 200 µL of strong sulphuric acid down the side of the test tube or microplate. For ten to twenty minutes, let the mixture rest at room temperature while the acid layer forms the bottom layer without mixing. The emergence of a yellow to orange colour in the top phenol layer indicates the presence of carbohydrates; use a spectrophotometer to measure the absorbance at 490 nm. Measure the absorbance of a standard glucose solution at different known concentrations to produce a calibration curve. Using the calibration curve derived from the reference glucose solution, determine the amount of carbohydrates present in the seaweed sample. The protocol has been carried out and replicated three times. The means ± SD

were used to express the data.

Extracting and estimating total lipid by gravimetric analysis method:

Take 100 mg of the lyophilised algal biomass. Transfer it to the glass vials after adding a chloroform:methanol (2:1, v/v) mixture to the tube for five minutes, sonicate the mixture for 20 minutes, incubate the mixture at room temperature. After sonicating the mixture, pour the supernatant into a tube and centrifuge it for ten minutes at 2000 g. After transferring the supernatant to the preweighed beaker, heat it in the oven at 55°C for the entire night. After the solvent has evaporated, weigh the beaker and record the results in triplicate (n=3) using gravimetric analysis to measure the system's extracted lipids.

Metabolite profile and total compound distribution:

- Algal biomass was ground into a fine powder in order to prepare for the metabolite profiling analysis. For extraction, 100 mg of the dried sample was ground into a fine powder using a sterile mortar and pestle. One millilitre of a (1:2) chloroform: methanol of HPLC-grade was then used to suspend ten milligrammes of the fine powder. For 30 minutes, the mixture was vortexed and sonicated with periodic cooling to ensure that the bioactive components were effectively extracted. Samples were incubated in a 65°C water bath for 30 minutes. After centrifuging at 14,000 rpm for 10 minutes at room temperature (RT), The methanol supernatant was collected in another fresh sterile Eppendorf tube and kept for overnight at 50 °C for drying. the supernatant was collected and kept at -20°C for further analysis. The extraction process was repeated 2–3 times to ensure complete metabolite recovery.
- In molecular mass profiling, derivatisation using a silylation reagent and incubation at 60°C were employed to enhance the identification of a variety of metabolites. The samples were analysed using a Shimadzu GC-MS system equipped with an electron ionisation (EI) source and autosampler (Paidi et al., 2017). Under an optimum temperature program, gas chromatographic separation was performed using helium as the carrier gas in a silica capillary column. Several biological components were found using mass spectrometry in the 40–500 m/z range. Among the important biochemical groups into which the identified substances were divided were amino acids, organic acids, sugars, fatty acids, hydrocarbons, and other organic molecules. A bar diagram was used to illustrate the biochemical diversity of the algae, and peak area percentages were used to determine their relative abundances. All analyses were performed in triplicate, and statistical significance was defined as a p-value of less than 0.05.

We have identified 57 components in the entire metabolic profile of the chosen seaweed sample. The whole distribution of compounds by their occupied area, as determined by GC-MS analysis, is shown in this pie chart, which is divided into distinct groups. Hydrocarbons are represented by the grey segment, organic acids by the yellow segment, organic compounds by the green segment, sugars by the dark blue segment, and amino acids by the blue segment as illustrated in figure 2.

The existence of complex bioactive molecules is indicated by the observation that Organic Compounds (OC) make up the biggest portion of the overall compound distribution. Hydrocarbons (HC) and organic acids (OA) are also abundant and greatly contribute to the chemical composition of the sample. There is a moderate representation of sugars (S) and fatty acids (FA). In comparison to other constituent classes in this dataset, amino acids (AA) comprise the smallest percentage, indicating their relative rarity.

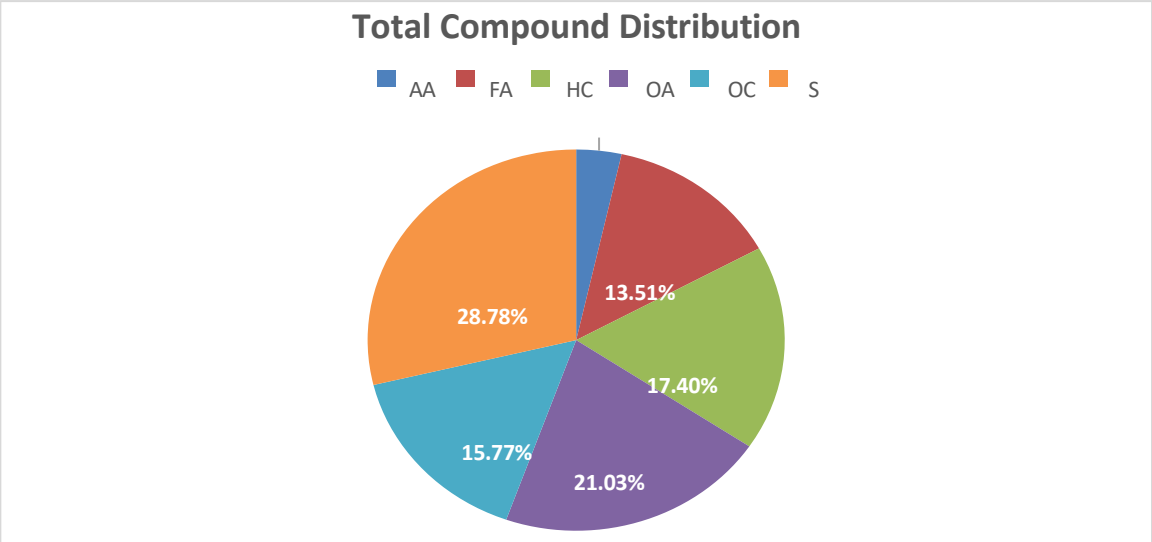

Figure 2: Metabolite profile of selected sample

Results and Discussion:

Ceramium, the red seaweeds that were collected, was shown to be usual along the shores of Mul Dwarka. As a result, *Ceramium* was selected and its anatomical characteristics were examined.

Figure 3: Morphological representation of red algal sample

Ceramium is a red alga with a bushy appearance that is connected to the substrate via the rhizoid, as we can see in figure 3.

Extraction and estimation of chlorophyll by using methanol:

Both the graph in figure 4 and table 1 show the concentrations of chlorophyll-a and chlorophyll-d. The vertical axis displays the concentration of these pigments in ug/ml. The scale has a range of 0–6 ug/ml. There are two bars on the graph; the first bar's green hue represents chlorophyll-a. It shows a much higher concentration of about 5 ug/ml. The measurement's fluctuation or margin of error is also displayed via an error bar at the top of this column.

ALGAL sample	Chlorophyll-a µg/mL	Average µg/mL	SD	Chlorophyll-d µg/mL	Average µg/mL	SD
R3a	5.08			0.22		
R3b	5.43	5.02	0.44	0.27	0.22	0.45
R3c	4.54			0.18		

Table 1: Tubular representation of concentration of chlorophyll

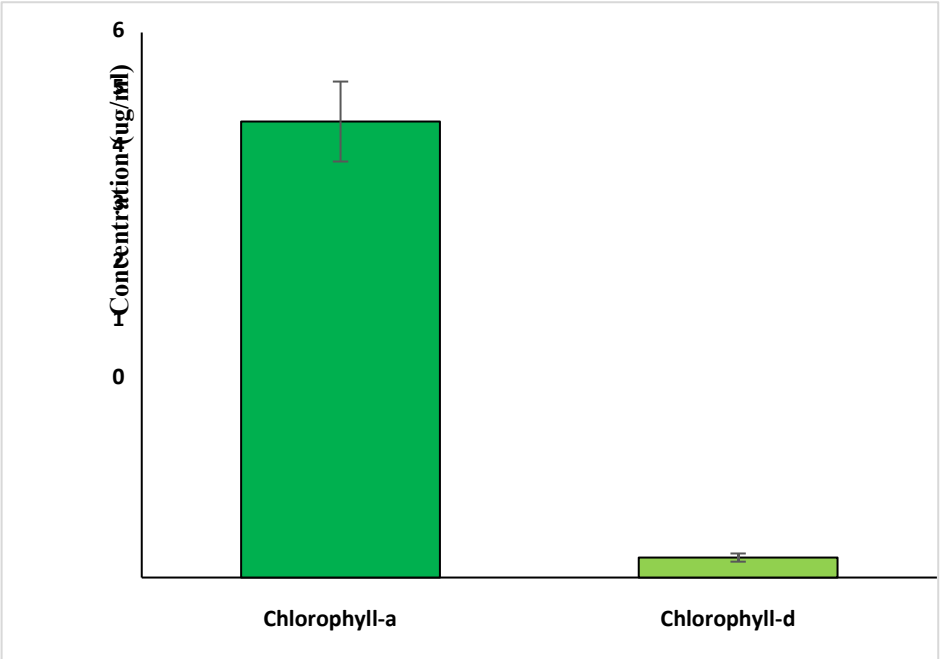

Figure 4: Graphical representation of concentration of chlorophyll

The second bar's light green hue represents chlorophyll-d. This concentration of less than 1 ug/ml is displayed with a somewhat shorter bar. The sample includes substantially more chlorophyll-a than chlorophyll-d, as seen by the sharp height difference between these two bars.

Estimating total carbohydrate by phenol-sulfuric acid method: The phenol-sulfuric acid technique has been used to determine total carbohydrates. The top phenol layer has developed a yellow to orange hue, which indicates the presence of carbohydrates. Once the colour has developed, use a spectrophotometer to measure the solution's absorbance at 490 nm, an appropriate wavelength. The findings are shown in table 2 and figure 5. The aforementioned approach has been used to estimate 9% of the total carbohydrates.

ALGALSample	Carbohydrate Concentration (%)	Average (%)	SD
R3a	7.33	9 (plus or minus 1.62)	1.62
R3b	10.57		
R3c	9.1		

Table 2: Tubular representation of concentration of carbohydrate

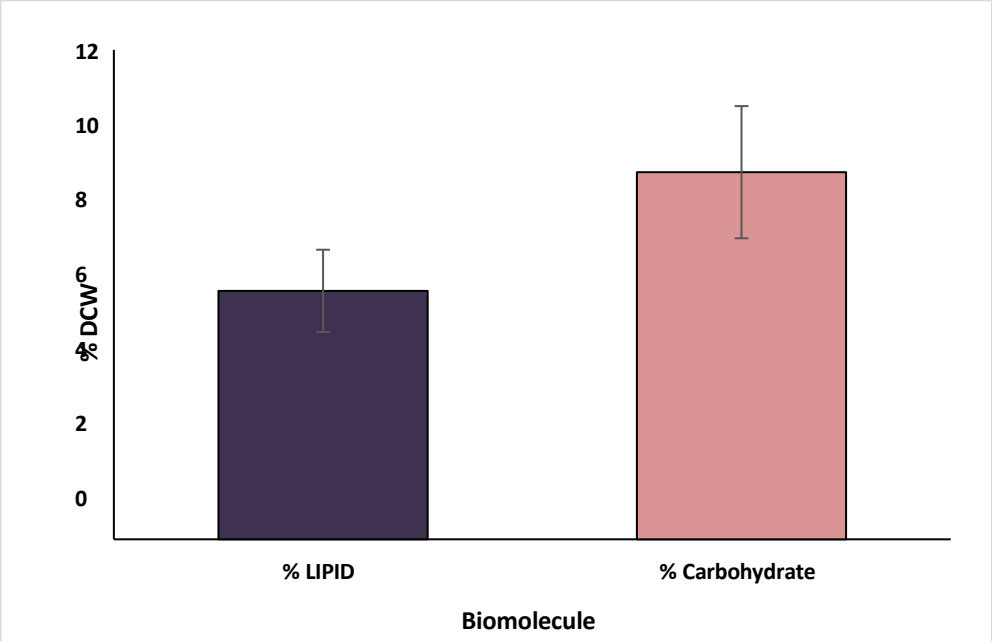

Figure 5: Graphical representation of lipid and carbohydrate content Estimating total lipid by gravimetric analysis method:

Lipid was calculated using the gravimetric analysis technique, which involves evaporating the solvent to quantify the extracted lipids of the system by weighing the beaker and recording the findings in table 3. This approach has been used to estimate 6.09 percent of the total lipid.

ALGAL Sample	LIPID Concentration (%)	Average (%)	SD
R3a	5.09	6.09	1.01
R3b	6.07		
R3c	7.11		

Table 3: Tubular representation of concentration of lipid lipid and carbohydrate content Metabolite profile and total compound distribution:

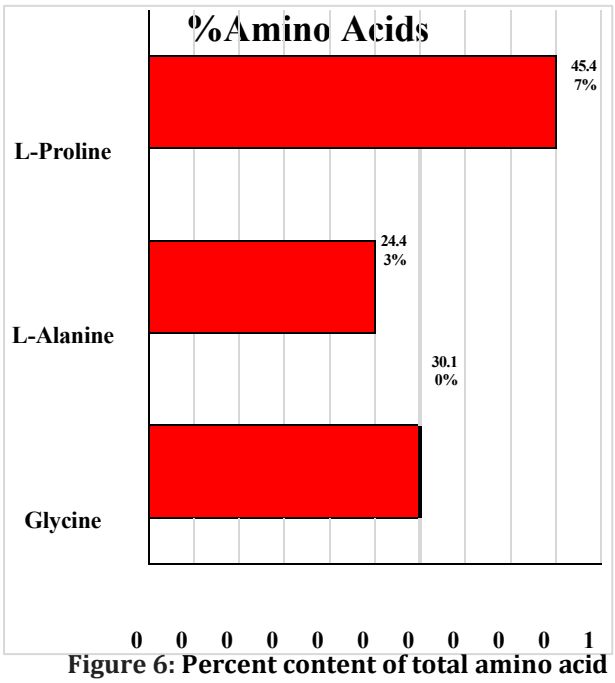

Figure 6: Percent content of total amino acid

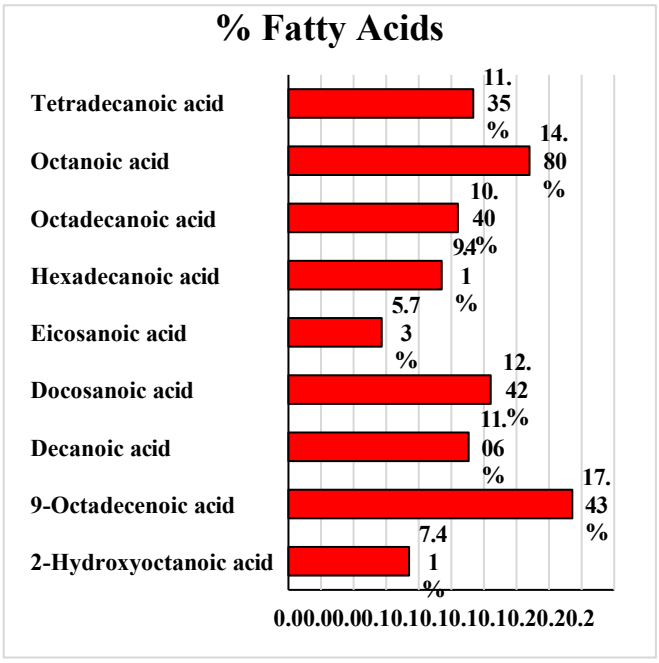

Figure 7: Percent content of total fatty acids

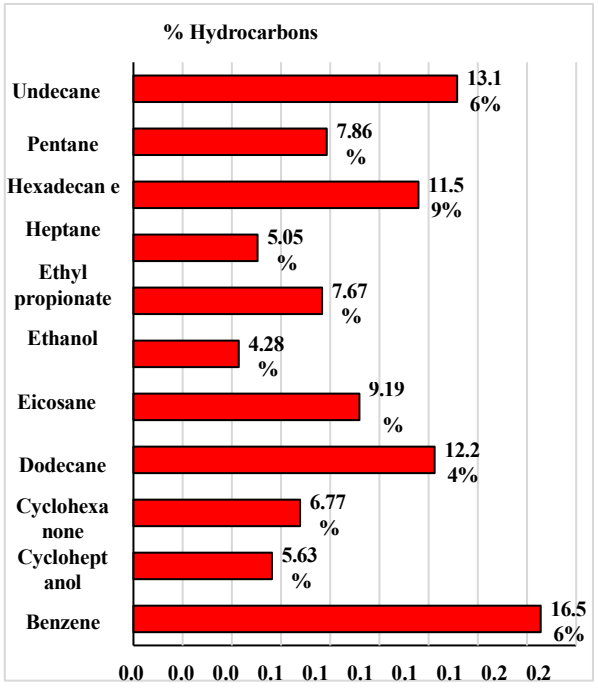

Figure 8: Percent content of total hydrocarbons

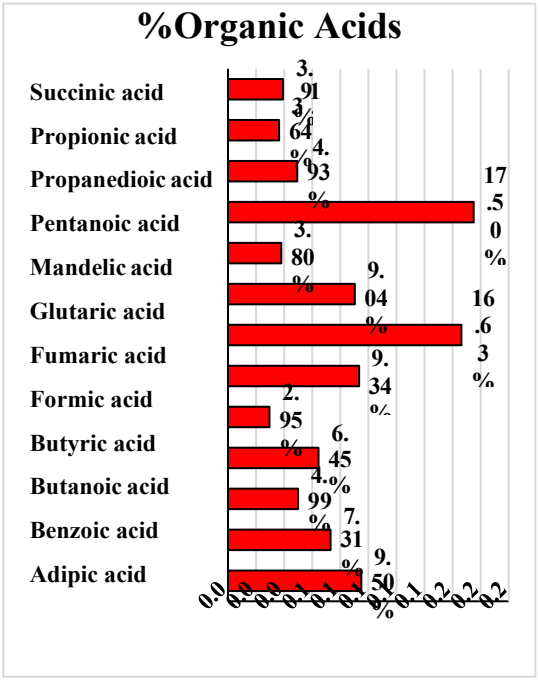

Figure 9: Percent content of total organic acids

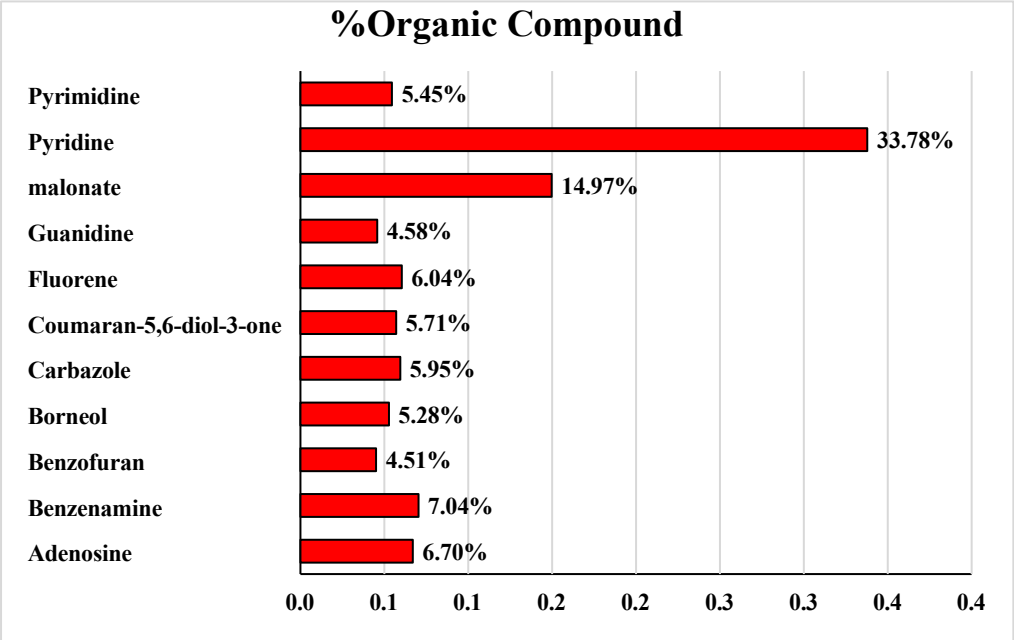

Figure 10: Percent content of total organic compound

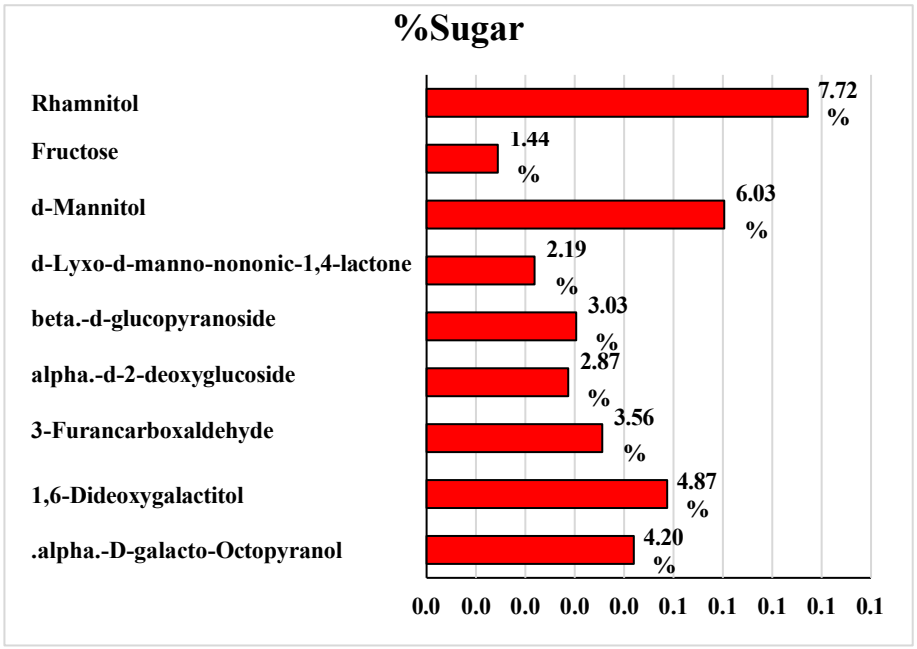

Figure 11: Percent content of total sugar

The presence of active substances was determined by GC-MS. An essential tool for biomolecule profile of pathway analysis is the metabolite profile. Red seaweed's richness in amino acids, organic acids, fatty acids, hydrocarbons, sugar, and other organic components is highlighted by this percentage bar diagram in figure 6 to 11, which validates the algae's varied biochemical profile by percentage. Three amino acids L-alanine, L-proline, and glycine as well as several fatty acid components found in a red algal sample by GC-MS analysis are shown in the bar graph along with their relative abundance (%).

We can see in figure-6 that, L-Proline is the most abundant amino acid in the chart with 45.47% among all amino acids. Figure-7 represents 9-Octadecenoic acid 17.43% highest percentage among the listed fatty acids. The most prevalent hydrocarbon found is benzene that represented in Figure-8. There is a mixture of cyclic molecules (like cyclohexanone), alcohols or esters (like ethanol, ethyl propionate), and straight-chain alkanes (like undecane, dodecane). The organic acid profile in Figure-9 is dominated by fumaric and pentanoic acids. The presence of a variety of short-chain, dicarboxylic, and aromatic acids points to a sample

with a broad metabolic or biochemical origin, maybe from a natural extract such as algae, microorganisms, or plant matter. The organic component profile is dominated by pyridine, for over one-third of the content as showen in figure-10. Heterocyclic substances (pyridine, pyrimidine, carbazole), aromatic compounds (benzenamine, fluorene), and bioactive molecules (adenosine, guanidine) are all included in the chart. Figure-11 represents Rhamnitol and d-Mannitol, both sugar alcohols (polyols), are the most abundant compounds. Mixture of monosaccharides, sugar alcohols, and possibly sugar derivatives are observed in the chart.

Metabolic pathway affected by the compounds:

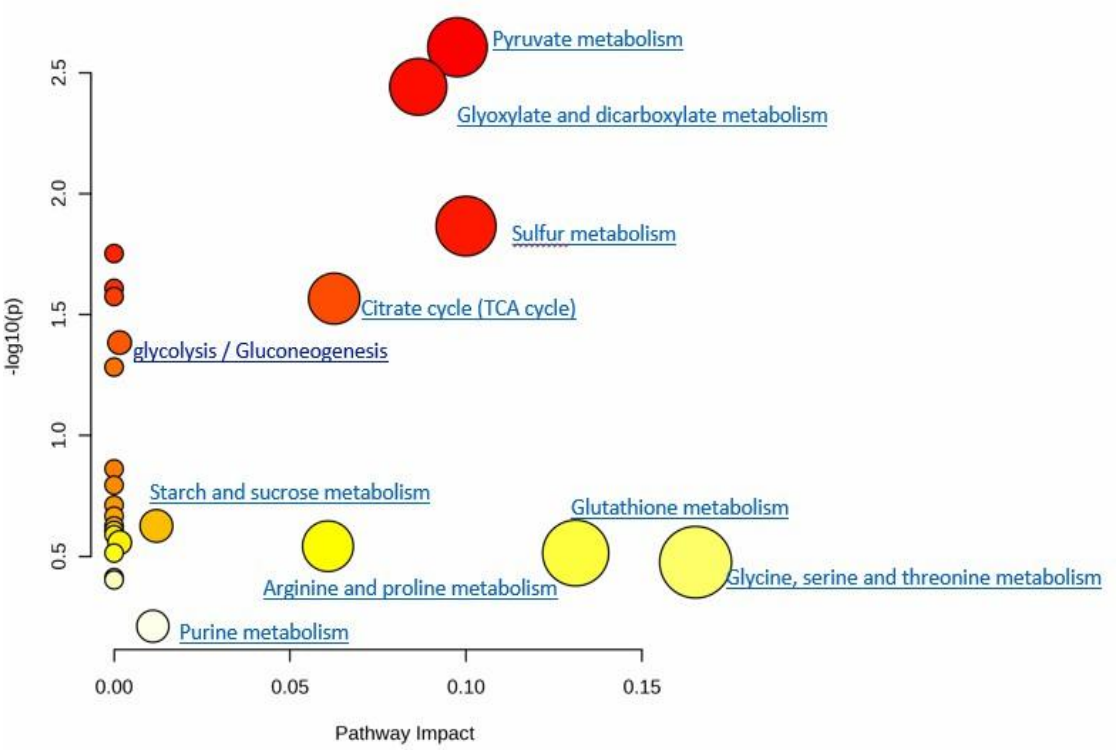

Figure 12: Pathway impact: metabolic pathway affected by the compounds

The metabolic pathways impacted by the chemicals found in the GC-MS data are shown by each marked pathway in figure 12. The Y-axis displays the influence of each route according to the metabolite, while the X-axis displays the statistical significance of the pathway. The quantity of metabolites is indicated by the bubbles. More metabolites are implicated when the bubbles are larger. The bubbles of orange colour are for intermediate, yellow for moderate, and red for high significance indicate the degree of importance.

Energy metabolic processes, such as glycolysis, the TCA cycle, and pyruvate metabolism, have a major impact and significant. The metabolism of amino acids, such as glutathione, arginine, proline, glycine, and serine, has a modest level of significance. There is less of an impact on purine metabolism and the metabolism of carbohydrates (such as starch and sucrose).

The oceanic environment, the planet's biggest ecosystem, provides a wealth of neglected potential for research and commercial applications. This study highlights the importance of marine resources for a wide range of sectors; in particular, we focused here is on red algae. Numerous new substances generated by various species, such as enzymes, hormones, metabolites, and so on, that were effectively obtained from the marine environment in earlier research.

This study supports red algae's biochemical and industrial potential, especially with regard to its nutritional makeup and profile of bioactive metabolites. The discovery of R-phycoerythrin and other useful substances creates new opportunities for long-term uses in medicine and cosmetics that we have discussed and mentioned in previous manuscripts. To investigate scalable extraction techniques and improve the economic feasibility of bioactive compounds obtained from rhodophyta we need to dive in for more investigation.

Conclusion:

Significant amounts of lipids, carbohydrates, and chlorophyll were found in the red seaweed samples that were obtained after biochemical examination. These elements are essential to metabolic processes and add to the seaweeds overall nutritional content. One important component that is essential to the

production of algal cell walls and energy storage is carbohydrates. The metabolomic profile of Rhodophyta was better understood thanks to GC-MS analysis, which also revealed a wide range of bioactive substances, including as sugars, amino acids, organic acids, fatty acids, hydrocarbons, and other organic metabolites. These molecules existence raises the possibility of a number of uses, especially in the food, cosmetics, and pharmaceutical sectors. Significant amounts of fatty acids and organic acids have been found to have anti-inflammatory and antioxidant qualities. Red algae's commercial relevance is further enhanced by hydrocarbons, which are frequently associated with energy storage and metabolic processes.

Furthermore, optimisation is necessary for the large-scale extraction of bioactive chemicals in order to guarantee effective yield and sustainable harvesting. Subsequent research ought to concentrate on sophisticated extraction processes, extensive cultivation strategies, and a more thorough examination of the functional characteristics of these bioactive metabolites.

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Declaration of Conflicting Interests:
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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