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Sustainability Spotlight Statement

Marine ecosystems, though vast and biodiverse, remain underutilized despite their immense potential to support emerging food resources and strengthen the blue economy. Seaweeds such as *Sargassum cinereum* offer sustainable alternatives to synthetic additives through their rich nutritional profile and diverse bioactive molecules, directly supporting goals related to health, nutrition, and responsible production. With rising global demand for natural antioxidants, optimizing green extraction methods like ultrasound-assisted extraction enables efficient, low-impact utilization of marine biomass. Exploring and valorizing abundant macroalgae along the Karnataka coast aligns strongly with SDGs on zero hunger, good health, sustainable industry, and conservation of ocean resources, promoting a balanced and future-ready marine-based economy.

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Optimization of *Sargassum cinereum* bioactives extraction parameters using ultrasound-assisted extraction with insights into chemical and nutritional composition

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Abstract

Marine organisms, particularly macroalgae, are globally recognized as key sources of valuable biomolecules with applications in the agricultural and pharmaceutical sectors. They are also an inexpensive source in developing countries with high population density, such as India, where pressing demands conflict with the sustainable use of resources. The abundance of *Sargassum* spp. in India could provide an opportunity to create cost-effective marine-derived natural products, and the implementation of an optimized extraction and scaling process could contribute to overcoming sustainability issues. Thus, the present study addressed the nutritional, mineral, and fatty acid composition of *Sargassum cinereum* J. Agardh from the Karnataka coast. Ultrasound-assisted extraction (UAE) was also used for optimizing bioactives yield using Response Surface Methodology (RSM) with Box-Behnken design (BBD). Carbohydrates were the most abundant (53.15%) macronutrients, followed by fiber (20%) and protein (13.35%) on dry weight basis. Fatty acid composition showed the abundance of palmitic acid, with a varied mineral composition, similar to other macroalgae already in use as a feed ingredient. RSM-BBD analysis underlined the impact of solvent concentration, time, including solid-to-solvent ratio, on the yield of total phenolics and flavonoids content, and antioxidant activity. Optimized parameters clearly evidence the interaction effect, presented through second-order polynomial equations. Although differences may be stated depending on sample processing techniques, geographical variability, storage

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conditions, and possible degradation of sensitive compounds, the data obtained here raise interest in future studies, given the potential of *Sargassum* spp., which grows abundantly and is nutritionally and chemically rich, and can be highly valued and used for multiple purposes.

Keywords: Response Surface Methodology; Marine macroalgae; Phytochemicals; Seaweeds; Ultrasound-Assisted Extraction.

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Introduction

Terrestrial ecosystems are far more explored and studied for their resources than marine habitats, which remain underexplored despite covering the majority (about 70%) of Earth's surface.[1] With the advances stated in new technologies and engineering, such as different types of vehicles, including manned submersibles, remotely operated, autonomous underwater, and hybrid vehicles, gliders, landers, profilers, and scuba diving, it has been possible to unlock the scientific exploration of marine environments. At the same time, the growing interest in natural products to replace synthetic ones towards health promotion and disease prevention has boosted the exploration of structurally diverse molecules from marine organisms for multiple purposes.[2-4]

Marine ecosystems are extremely rich in biodiversity and composed of a complex community of interacting organisms, including bacteria, protozoans, algae, chromists, fungi, animals, and plants.[5] Although the search for marine natural products has mainly focused on marine invertebrates, seaweed has become a cheap source of great interest due to its abundance of bioactive compounds with high potential. Their ability to withstand adverse conditions, such as exposure to variations in salinity, pressure, light, and nutrient availability, together with the defense mechanism developed through chemicals against predation, makes them highly interesting to produce structurally diverse and innovative bioactive compounds.[6,7] In the marine environment, there are mainly two types of algae: microalgae (microscopic and mostly planktonic) and macroalgae, which are macroscopic and benthic in nature.[8]

Marine macroalgae, also called seaweeds, are classified into green, brown, and red algae due to their size, multicellular organization, and attachment to substrata.[9] There are 1200 green, 2000 brown, and 6000 red macroalgae globally, linked to an estimated annual production of 6,756,521 million tons of biomass, with the global market value amounting to US$5.5–6 billion per year. As

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India has a long coastline, around 11,098 km, with a 2.5 million km^2 economic zone and 0.13 million km2 shelf area, it provides an opportunity for the blue economy, which has gained global attention.[10-13] Among these, the Karnataka region covers 343 km of coastline in the West Central region of India, including estuaries and accessible coastlines. A recent study by Chippalakatti et al. [14] revealed that *Sargassum*, *Gracilaria*, and *Ulva* spp. are abundant marine macroalgae in Karnataka with huge economic potential. They are a significant source of minerals, proteins, carbohydrates, vitamins, fatty acids, and numerous valuable compounds, such as ascorbic acid, carotenoids, tocopherols, chlorophyll derivatives, polyphenols, phlorotannins, and mycosporine-like amino acids.[15] Particularly, *Sargassum* spp. have gained increased research interest because of their richness in bioactive molecules, such as sargaquinoic acids, sargachromenol, and pheophytine, which are esteemed for their antioxidant effects and potential applications in functional foods, nutraceuticals, and natural food preservatives.[16,17] The demand for natural antioxidants is rising amid concerns about the health risks of synthetic additives, and the growing consumer shift towards sustainable alternatives, further making it an industrial and scientific priority to identify and optimize their extraction process.[18]

The blue economy not only underlines the economic potential of the marine ecosystem but also recommends ensuring the responsible and equitable exploitation of oceanic resources.[19] Optimization of extraction procedures for bioactive compounds like phenolics and flavonoids warrants sustainable resource utilization as well. Ultrasound-Assisted Extraction (UAE) is one among the emerging modern techniques that can reduce solvent consumption, to give higher yield within shorter time duration with preserved bioactivity, making it a sustainable alternative to conventional extraction techniques.[20,21] Thus, the present study focuses on *Sargassum cinereum* J. Agardh, an abundantly distributed brown macroalga along the rocky intertidal regions of the

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Karnataka coast, where high standing-stock populations have been reported (Chippalakatti et al. 2025). Despite the growing research interest in several *Sargassum* species, comprehensive studies addressing the nutritional profiling, fatty acid composition, and systematic optimization of bioactive extraction from *S. cinereum* remain limited. Given its regional abundance and potential for sustainable biomass availability, scientific evaluation and process optimization are essential to support its valorization in nutraceutical, functional food, and feed applications. Therefore, the present study investigates its chemical and nutritional composition and fatty acid profile, along with the optimization of bioactive extraction using Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD).

Experimental

Marine macroalgae collection, identification, and processing

As part of a diversity study, marine macroalgae were collected from the rocky shores of coastal Karnataka between 2022 and 2023.[14,22] Among the specimens collected, *Sargassum* sp., a brown alga, was found to be growing abundantly at Hadin Eco beach, Bhatkal, Uttara Kannada, Karnataka, India (latitude: 13.949164; longitude: 74.548845; Figure 1). The idea of adding value to an abundant natural resource led us to explore the economic potential of *Sargassum* spp., prompting us to collect large quantities of its biomass and transfer it to the laboratory in an insulated ice box. The sample was washed in running tap water to remove debris, sand particles, and epiphytes. Morphological observations were made, and a herbarium was prepared and deposited at Karnatak University in Dharwad, Karnataka, India. The marine macroalga was identified as *Sargassum cinereum* J. Agardh. The sample was shade-dried for a week and then

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made into powder using a mechanical blender. Macroalgal powder was then packed in an airtight container for further analysis.

Chemicals and reagents

All solvents, such as methanol, ethanol, chloroform, and hexane, used in the study were of analytical grade. Chemicals, namely aluminium chloride, boron trifluoride, hydrochloric acid, nitric acid, perchloric acid, potassium hydroxide, sodium carbonate, sodium hydroxide, and sulphuric acid; standards, such as ascorbic acid, gallic acid, quercetin, and minerals along with the reagents, like bromocresol green, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu (FC), molybdenum blue, and zirconium oxychloride were brought from HiMedia Laboratories, Mumbai, India and Sigma-Aldrich, Bengaluru, India. Double-distilled water was used throughout the study for chemical preparation.

Nutritional and mineral composition of S. cinereum

Nutrient composition, including protein, lipid, carbohydrate, ash and moisture, was analyzed using standard AOAC guidelines with slight modifications. After the analyses, Atwater factors were used to calculate the energy value in kcal/100 g. Moisture content was determined by taking a pre-weighed fresh sample, drying it at 105 °C in an oven until it attained a constant weight. Weight differences were used for calculations. Ash content was analyzed using the AOAC.[23] protocol. Briefly, 2 g of dried macroalgal sample was placed in a preheated silica crucible and kept at 550 °C for 4 h in a muffle furnace, after which it was cooled in a desiccator. Residual weight was used to calculate the ash percentage. Crude protein content was calculated by multiplying the conversion factor of 6.25 by the total nitrogen quantity, as determined by the Kjeldahl distillation

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method.[24] Crude lipids were extracted from the macroalgal powder using a Soxhlet apparatus with a chloroform: methanol solvent mixture at a ratio of 2:1 (v/v), as described by Wong and Cheung.[25] Then, the contents of crude lipids were gravimetrically determined after oven-drying at 80 °C. Crude fiber content of the sample was determined by digesting the sample with 1.25% H_2SO_4 and 1.25% NaOH, according to the AOAC.[23] protocol. The sample was dried in an oven at 105 °C, then the ash was obtained by burning it at 525 °C in a muffle furnace overnight, after which it was cooled in a desiccator. Weight differences were then noted and calculated. The results of these parameters were then converted to a fresh weight basis and used to calculate the carbohydrate content by subtracting the sum of the percentages of ash, moisture, protein, fiber, and lipid in hundredths, as given in Wirenfeldt et al. [26]

Mineral Composition of S. cinereum

Mineral composition was determined by the sample's acid digestion. The major minerals, like sodium and potassium, were quantified using a flame photometer, as reported by Manivannan et al. [27]. Phosphorus was determined based on molybdenum blue coloration using the spectrophotometric method. Magnesium and calcium were determined by titration using bromocresol green and zirconium oxychloride solution, following the method described by Yadav [28]. Other minerals, such as copper, zinc, iron, and manganese, were analyzed using an atomic absorption spectrophotometer (NOVA 400, Analytic Jena, Jena, Germany), equipped with single hollow cathode lamps and an air-acetylene burner, using respective standards.[29]

Fatty acid profiling by gas chromatograph and flame ionization detector

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Lipid extracted for nutrient analysis was subjected to esterification, converting the fatty acids into methyl FAME as per the AOCS [30] protocol, with modifications in the quantity of sample as follows: 150 mg of lipid was subjected to saponification by adding 3.75 mL of 0.5 N methanolic KOH, followed by transesterification using 3 mL boron trifluoride–methanol, incubated at 60 ºC for 30 min, and instantly cooled for 5 min in an ice bath. The solution was mixed with 1 mL each of hexane and distilled water and vortexed. Then, the undisturbed upper layer containing methyl esters was collected in GC vials. Analysis was carried out using a GC Clarus 480 PerkinElmer equipped with a silica capillary column (60 m × 0.25 mm, I.D., 0.25 μm film), and a FID, as described by Berneira et al. [31]. The operation features, such as the injector with detector temperature, were set to 250 ºC. The temperature of the column was initially set at 80 ºC for 2 min, then increased to 200 ºC, over 10 min at a rate of 6 ºC/min, and further increased to 220 ºC, over an additional 30 min at a rate of 2 °C/min. FAMEs were analyzed using a GC equipped with a FID. Identification of individual FAMEs was performed by comparing their retention times with those of a certified multi-component FAME reference standard analyzed under identical chromatographic conditions.

Phycochemical Extraction by UAE from S. cinereum

Ethanol was selected as the solvent without any preliminary screening due to its eco-friendly, less hazardous, and more convenient nature.[32] Different quantities of macroalgal powder were ultrasonicated in an ultrasonic water bath for different times and at different concentrations of ethanol, as mentioned in the optimization experimental design. Ultrasonicated samples were filtered using a muslin cloth, followed by 10 min of centrifugation at 5000 rpm. Supernatant was collected and stored at 4 °C for further analysis.[33,34]

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Experimental design for UAE optimization

Extraction efficiency is influenced by multiple operational and material-related factors, including ultrasonic intensity, frequency, temperature, pressure, and sample characteristics such as particle size and moisture content.[35,36,37] Nevertheless, solvent concentration, extraction time, and solid-to-solvent ratio remain the most universally impactful variables across extraction methods and are therefore considered critical parameters for optimization. In the present study, independent variables such as solvent concentration (X_1), time (X_2), and solid-to-solvent ratio (X_3) were considered for optimization phycochemical extraction process. The experimental ranges of the selected variables were determined based on published literature and prior laboratory investigations on other macroalgae Thangadurai et al. [38] These limits were chosen to encompass a statistically meaningful and practically feasible operating window, enabling effective bioactive extraction under controlled extraction time and solvent usage. Other parameters, such as temperature, were held constant, as was the ultrasonic frequency (50 kHz), ultrasonic power (60 W) and 100% amplitude. Optimization was conducted using a three-factor, quadratic, randomized BBD model in RSM, with the aim of maximizing the responses, such as total phenolic content (Y_1), total flavonoid content (Y_2), and antioxidant capacity (Y_3), based on the designs of Liyana-Pathirana and Shahidi [39] and Zhang et al. [40] with slight modifications. Variables were fixed at three levels, coded as -1, 0, and +1, and the lower and upper limits of the variables were chosen to maximize the extraction of phenolic and flavonoid compounds, and their DPPH inhibition capacity from a smaller sample quantity in a shorter time period. Table 1 summarizes the natural and coded values for all three independent variables used in the experiments. The experimental matrix, along with its response values, was recorded in the results and discussion section. The extraction yield

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was analyzed using BBD of response surface regression (Eq. 1) and fitted to a second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j$$ ----------Eq. **(1)**

Where, Y = predicted responses, such as total phenolics, total flavonoids, and DPPH inhibition %; β_0 = constant coefficient; β_i = linear coefficient; β_{ii} = quadratic coefficient; β_{ij} = cross-product coefficient; X_i and X_j = independent variables. The model was validated by comparing experimental and predicted values.

Total Phenolic Content Analysis

The total phenolic content (TPC) of *S. cinereum* extracts obtained was determined using the Folin-Ciocalteu method described by Wolfe et al. [41] with minor modifications to the quantities of reagents and extracts used. Briefly, reaction mixtures were prepared by adding distilled water (50 µL), extract (12.5 µL), and Folin-Ciocalteu reagent (12.5 µL) were added to a 96-well microtiter plate. After incubating the reaction mixture for 10 min at room temperature, 7% saturated sodium carbonate (125 µL) and distilled water (100 µL) were added. The mixture was then incubated again for 90 min in the dark at room temperature. After the color developed, the absorbance (OD) was measured at 760 nm in a 96-well microtiter Multiskan Sky spectrophotometer (Thermoscientific). Gallic acid was used as a standard phenolic compound to obtain a calibration curve. The TPC of the extracts was then calculated and the results expressed as milligrams of gallic acid equivalents per gram of dry weight of the sample (mg GAE/g DW).

Estimation of Total Flavonoid Content

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Total flavonoid content (TFC) of the extracts was analyzed using the protocol by Madar et al. [42] which is a modified version of Luximon-Ramma et al. [43] protocol. In brief, 1.5 mL of 2% methanolic $AlCl_3$ was mixed with 1.5 mL of the algal extract, shaken vigorously to ensure proper mixing and then incubated for 10 min in the dark at room temperature. Absorbance was then measured at 367 nm using a 96-well microtiter Multiskan Sky spectrophotometer. The calibration curve obtained for the standard quercetin was used to determine the TFC of the sample, and the results were expressed as milligrams of quercetin equivalents per gram of dry weight of the sample (mg QE/g DW).

Antioxidant Assay by DPPH Inhibition

The antioxidant activity was assessed using the DPPH inhibition method adopted by Magdum et al. [44]. In brief, a reaction mixture was prepared by adding 10 µL of each extract to 290 µL of the DPPH solution, which was subjected to incubation at room temperature for 30 min in the dark. Absorbance was then read at 517 nm using a 96-well microtiter Multiskan Sky spectrophotometer. A decrease in absorbance of DPPH by both the extract and the control (DPPH solution with methanol in the case of the extract) was noted. The DPPH inhibition % of each extract was analyzed by comparing its absorbance with that of the control using Eq. (2).

$$DPPH\ inhibition\ \% = \frac{(Absorbance\ of\ control - Absorbance\ of\ test\ sample)}{Absorbance\ of\ control} 100 \quad \text{----------Eq. (2)}$$

Statistical Analysis and Software Tools

Proximate and mineral composition analyses were carried out in triplicate, and results are expressed as mean ± standard deviation done using Excel. Design Expert software version 13 (Stat-

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Ease, Inc., Minneapolis, MN, USA) was used for the optimization experiment, regression analysis, and statistics involved in the RSM-BBD case. The same software was also used to develop all the graphs and pictures.

Results and discussion

The present study aimed to analyze the chemical and nutritional composition, including mineral and fatty acid profiles of the brown seaweed *S. cinereum*, an abundant natural marine resource from the coastal Karnataka, India. The main objective of this study was to optimize the extraction process for bioactive components. The independent parameters chosen were solvent concentration (medium-dependent parameter) X_1 (%), time of extraction (equipment-based parameter) X_2 (min), and the solid-to-solvent ratio (matrix-dependent parameter) X_3 (mg/ml), as these are well-known factors that greatly affect the quantity of bioactives extracted by each extraction technique. Updated techniques, like pressurized liquid extraction, enzyme-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and UAE, have more advantages than disadvantages, especially UAE, which has gained attention due to its extraction efficiency, minimal solvent use and thermal degradation, and cost-effectiveness.[45] The study conducted an optimization experiment to recover bioactives, such as phenolic and flavonoid compounds having DPPH-inhibiting capabilities, using ethanol as a solvent. This study employed mathematical modelling using the Box-Behnken design of surface response methodology. A total of 17 experimental runs at five central points with different combinations of independent variables were carried out, for all three responses, such as TPC (Y_1), TFC (Y_2), and DPPH inhibition % (Y_3).

Nutrient and Mineral Composition of S. cinereum

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Determination of the nutritional and mineral composition of every organic matter is important. Specifically, to what concerns to marine macroalgae, the composition varies depending on species, season, temperature, climate, geographic area, water, and even sea conditions. *S. cinereum* collected from the Karnataka coast has a balanced composition of nutrients and minerals, as shown in Tables 2 and 3. The results of the present study revealed that *S. cinereum* has a moisture content of about 80.2%. Of all the nutritional components, carbohydrates are the most abundant, accounting for 10.52% of the wet weight, followed by fiber at 3.96%. Similar quantities of protein (2.48%) and ash (2.64%) were also recorded, whereas lipids were the least abundant component (0.19% of the wet weight). The nutritional profile obtained in this study aligns with previously reported values for *Sargassum horneri* and other brown macroalgae reported by Murakami et al. [46] and Premarathna et al. [47]. In addition, the estimated energy value (274.73 kcal/100 g DW) is consistent with the range reported for *Sargassum tenerrimum* and *Sargassum asperum* [48], indicating comparable calorific potential among these taxa.

Regarding mineral composition, calcium was the most abundant macro mineral with 40 mg/g, suggesting its potential as a natural supplement. Nitrogen was the second most abundant (22.1 mg/g), indicating the presence of a good quantity of protein. The remaining minerals, potassium, magnesium, sodium, and sulphur, were present in moderate but considerable amounts. Phosphorus is the least abundant mineral (0.239 mg/g). In the case of micro minerals, iron was found to be present at a relatively high level (9.71 mg/g), followed by manganese (7.88 mg/g). Copper and zinc were present in moderate quantities, whereas boron was present in trace amounts (0.48 mg/g). These results highlight the importance of these minerals as feed and food ingredients. Higher iron levels support respiration in both animals and humans, while manganese and copper levels are within the functional range of enzyme activity and bone development. The sodium-to-potassium

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ratio was also within the tolerable range for maintaining electrolyte balance.[49,50] Despite their good nutritional composition, macroalgae are not widely accepted as human food. They are not economically under exploitation, due to their unfamiliarity, taste, texture, and availability, but they are gaining huge importance and attention in the animal feed industry.[51-53] Marín et al. [54] tested *Sargassum* spp. as a feed ingredient for sheep and recommended its use in tropical and subtropical regions, as it did not affect sheep's feed intake when incorporated at 50%. A recent report by Carrillo-Domínguez et al. [55] also suggested that *Sargassum* spp. biomass could be used as an animal feed ingredient due to its mineral, amino acid, fatty acid and bioactive compound content. However, it is recommended to be used in lower concentrations as it contains high levels of fiber, salts, and complex carbohydrates. The presence of toxic minerals should also be considered, and every natural sample should be analyzed unless it is artificially cultured.

Fatty Acid Composition of S. cinereum

GC-FID analysis was used to assess the fatty acid profile of *S. cinereum*. Palmitic acid, a saturated fatty acid (SFA), accounted for nearly half of the total composition (48.01%), followed by oleic acid, (17.38%) a monounsaturated fatty acid (MUFA). Palmitic acid, along with stearic and myristic acids, constituted 58.79% of the total SFA, indicating that stable lipids constitute a large portion of the sample. Polyunsaturated fatty acids (PUFA), such as linoleic, α-linolenic, and arachidonic acids, accounted for 6.01%, 4.30% and 6.33% respectively. These essential fatty acids highlight the nutritional, therapeutic, and industrial potential of *S. cinereum*. The clear dominance of SFA, followed by MUFA and PUFA, is a common trend reported in marine macroalgae. Similar results were observed in most *Sargassum* spp., with descending order of abundance for SFA,

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MUFA and PUFA, with most studies showing that palmitic and oleic acids are the predominant ones.[56,57]

Optimization of UAE Parameter for Bioactives from S. cinereum

Extraction Process Modeling

The actual values of the experimental design for each of the three levels of the experimental runs, along with the respective response results, including the values predicted by the model, are shown in Table 5. The TPC of *S. cinereum* ranged from 0.1649 to 1.14 mg/g GAE at experimental runs 7 and 11 but was predicted to be 0.2507 and 1.07 mg/g GAE, respectively. In the case of TFC, the amount varied from 0.9010 to 4.10 mg/g QE at the run numbers 3 and 8, respectively, where the model predicted values of 1.36 and 3.43 mg/g QE. Similarly, the inhibition percentage for the antioxidant activity by the DPPH assay ranged from 1.64 to 18.51% in the actual results and from 2.11 to 18.58% in the predicted results at runs 2 and 3. These experimental results were subjected to ANOVA and regression analysis, which helped to identify the effect of the parameters on the yield of responses using Design Expert software. Fit summaries of the second-order polynomial model showed that the linear model fits best for Y_1 and Y_2, which had an insignificant lack of fit, as required, with a low standard deviation of about 0.1233 and a regression coefficient (R^2) of 0.8614 for Y_1. The respective values were higher in the case of Y_2 at 0.6112 and 0.6033. Also, the quadratic model was the highest order polynomial with the maximum number of significant terms and an insignificant lack of fit for Y_3, with an R^2 value of 0.9761. ANOVA and regression analysis were performed on the experimental data to determine statistical significance. The results, along with the regression coefficients of the predicted second-order polynomial models, are summarized in Tables 6 and 7. The linear models for Y_1 and Y_2, with F-values of 26.93 and 6.59, respectively,

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implied the significance of the models. The pronounced effect of ethanol concentration X_1 and solid-to-solvent ratio X_3 on total phenolic content Y_1, can be found due to polarity mediated solubilization and mass transfer dynamics. Phenolic compounds encompass a wide range of structures with varying polarity, and hydroethanolic systems are widely recognized for enhancing their extraction by optimizing solvent polarity and improving cell wall permeability. [58,59] Adjusting ethanol concentration modifies dielectric properties of the solvent system, thereby influencing phenolic solubility and diffusion into the extraction medium. Furthermore, the solid-to-solvent ratio X_3 plays a critical role in establishing an effective concentration gradient between the algal matrix and solvent, thereby enhancing mass transfer and facilitating greater release of phenolic compounds. [35] An increased solvent volume relative to sample mass improves contact surface area and reduces saturation effects, leading to improved extraction efficiency. In contrast, total flavonoid content Y_2 was predominantly influenced by the solid-to-solvent ratio X_3. Flavonoids exhibit considerable structural diversity, including glycosylated and polymeric forms, which may display differential solubility behavior in hydroethanolic systems. [58] Consequently, solvent availability and diffusion efficiency become more critical determinants than solvent polarity alone. Thus, adequate solvent volume relative to biomass enhances mass transfer and facilitates improved recovery of flavonoid compounds. However, the DPPH inhibition percentage with the quadratic model has more significant terms, such as X_1, X_3, X_1X_3, X_1^2, X_2^2, and X_3^2. Kadam et al. [60] stated that an adequate fit is necessary to avoid poor results in response surface optimization. The required adequate precision required for this model is supposed to be greater than 4. The values of 16.957, 8.469, and 19.854 for Y_1, Y_2, and Y_3, respectively, ensure the good adequacy of the proposed model. Equations in terms of the actual factors were drawn up to describe the relationships between the independent factors and the response values in equations 3, 4, and 5.

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$$Y_1 = 1.59482 - 0.011981X_1 + 0.009929X_2 - 0.005900X_3$$ ---------- Eq. **(3)**

$$Y_2 = 2.8671 + 0.006104X_1 + 0.080417X_2 - 0.034130X_3$$ ---------- Eq. **(4)**

$$Y_3 = 7.65480 - 0.577575X_1 + 1.47100X_2 + 0.723499X_3 + 0.007003X_1X_2 - 0.003065X_1X_3 + 0.000348X_2X_3 + 0.003568X_1^2 - 0.096550X_2^2 - 0.004421X_3^2$$ ---------- Eq. **(5)**

Relationship among the Variables and Effects on Responses

Graphs illustrating the relationship between different variables could be drawn, including those showing single factors, interaction among the factors, contour plots, and 3D surface graphs. A negative linear effect was observed: as the concentration of ethanol increased, the TPC decreased significantly. This suggests that a moderate concentration of ethanol is optimal for extracting phenolic compounds, possibly due to a balance between polarity and solubility. Higher ethanol concentrations might reduce the solubility of polar phenolic compounds. The solid-to-solvent ratio showed a slight negative slope, i.e., higher solid loading reduces TPC yield, likely due to solvent saturation. In contrast, the curve was almost flat, indicating that extraction time (5-15 min), does not drastically influence TPC within this range. Shorter times may already be sufficient for phenolic extraction due to their solubility (Figures 2A-C). In the case of TFC, a positive correlation was observed with solvent concentration, indicating that flavonoids may require a higher ethanol concentration for efficient solubilization due to their moderate hydrophobicity. A positive linear effect was also observed, suggesting that these compounds require slightly longer for efficient diffusion; however, a negative slope was observed in the solid-to-solvent ratio for TFC, as was

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seen for TPC (Figure 2D-F). The DPPH inhibition (%) response was influenced by both interaction effects and individual variables. A quadratic response pattern was particularly evident in the interaction between time and solid-to-solvent ratio, where DPPH activity initially increased with increasing extraction time and solvent availability, followed by a decline beyond the optimal range. This trend may be attributed to the establishment of equilibrium between solute concentration in the matrix and solvent phase, limiting further enhancement in antioxidant recovery. DPPH activity decreased at higher ethanol concentrations, possibly due to reduced extraction efficiency of more polar antioxidant constituents. A similar trend was observed for total phenolic content. The decline in response at extended extraction time or higher solvent concentrations may be associated with reduced mass transfer efficiency or potential instability of certain antioxidant compounds under prolonged processing conditions. Contour plots (Figure 3) and 3D response surface graphs (Figure 4) were used here to visualize these interaction effects. Elliptical contours, particularly for the interaction between time and solid-to-solvent ratio, indicate a significant interaction effect, whereas less elongated patterns suggest comparatively weaker interactions. The response surface plots demonstrated that moderate ethanol concentration, optimal extraction time, and an appropriate solid-to-solvent ratio resulted in maximum DPPH inhibition, highlighting the presence of an optimal extraction window rather than a simple linear relationship. The desirability function confirmed these findings, supporting the effectiveness of response surface methodology in identifying the most favorable extraction conditions.

Optimum Parameters for UAE of Antioxidant Phenolics

All three response variables (Y_1, Y_2, and Y_3) were successfully optimized to extract the maximum quantity of TPC, TFC, and antioxidants in the shortest possible time using the parameters

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mentioned in Table 8, with additional confirmatory runs. The optimized extraction conditions predicted by the RSM model were experimentally validated, and the obtained values were in close agreement with model predictions. The percentage deviations between predicted and experimental values were 6.99% for TPC, 8.11% for TFC, and 9.51% for DPPH inhibition, indicating satisfactory predictive accuracy and confirming the reliability of the developed models. Although the highest yields of each response were obtained in different runs with different combinations of independent variables, this optimization process revealed that an ethanolic concentration of 40% with 36.35 mg/mL of material subjected to ultrasonication for 12.32 min could efficiently and reliably yield all three responses, i.e. TPC, TFC, and DPPH inhibition %. The experimental results showed 1.09495 mg/g GAE of TPC, 2.6289 mg/g QE of flavonoids, and 12.0711% DPPH inhibition, whereas the predicted values were 1.0234, 2.86115, and 13.3404, respectively with an overall desirability of 0.72 on a scale of 0 to 1, indicating that the chosen set of extraction conditions provides a fairly good compromise between maximizing all three responses simultaneously (Figure 5). Here, the TPC of *S. cinereum* extract under optimized conditions was lower than that of various Korean *Sargassum* spp. [61] The TPC obtained in the present study was higher than that obtained in previous research on *U. lactuca* conducted by Pappou et al. [62] and Amini et al. [63] but lower than that on *Ascophyllum nodosum*.[60] In relation to other food sources, the TPC and TFC are equivalent to the levels found in millets, as explained by Bheemaiah et al. [64] Furthermore, the % DPPH inhibition of the various runs with different combinations of independent variables in the present study falls within the range of *Sargassum horneri* extract.[65] However, the overall responses are comparatively lower, which may be due to various factors, such as the composition of the seaweed, which is not only dependent on the species, but also on its growth stage, maturity, environmental conditions at the sampling stations, season and other

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extraction conditions of the different methods and protocols used for estimation. Widyaswari et al. [66] noted that differential light exposure and nutrients influence the growth and chemical composition of seaweeds, leading to variations in bioactive substances, even within the same species. The coefficient of variation (CV%) ranged from 13.02% to 25.55% across the studied responses. Among them, the DPPH model exhibited the lowest CV% (13.02%) and a high R^2 value (0.976), indicating strong agreement between experimental and predicted values. Although TPC and TFC showed relatively higher dispersion, their adequate precision values (>4) and reasonable agreement between adjusted and predicted R^2 confirm the reliability of the developed models for optimization purposes. Overall, the method and models accurately predicted the optimal parameters for extracting antioxidant phenolics, eliminating the need for preliminary analysis in future extraction works.

Conclusion

Sargassum cinereum occurs abundantly in the coastal area of Karnataka, India. This natural marine resource has high potential for valorization as a useful product in the nutraceutical and animal feed industries due to its nutritional composition, which includes high levels of carbohydrates and proteins. Along with these, it could be a potent source of pharmaceuticals due to its bioactive and antioxidant properties highlight its potential for functional food and related value-added applications. Thus, optimization is a necessary step for economic viability. The response surface methodology effectively optimized UAE conditions, confirming that moderate solvent strength, extraction time, and solid-to-solvent ratio enhance the recovery of phenolic compounds and antioxidant activity. The developed models showed satisfactory predictive capability, particularly for antioxidant response, supporting their suitability for process optimization. These optimized

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parameters could be used as the standard for future economic levels of extraction. However, variations in the nutrient composition of marine macroalgae due to their higher absorption capacity with respect to geographic area and conditions may affect the potential utilization of brown macroalgae *S. cinereum* in functional food and feed industries. The currently identified limitations and gaps provide opportunities for future research.

Author contributions

P.C.: conceptualization, data curation, formal analysis, investigation, and writing the first version of the manuscript. D.T., M.N., N.C.-M. and P.M.: supervision, data curation, and methodology, writing – review and editing, methodology, conceptualization, resources. All authors reviewed and validated the final version of the manuscript.

Conflict of interests

The authors declare no conflict of interest.

Data availability statement

Any other external data are not used for this manuscript; all the data are generated from experimental analysis.

Ethics declaration

Not applicable

Acknowledgements

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683 **Table 1.** Coded values and actual values of the upper and lower limits of the independent variables
684 to be optimized for bioactives extraction.

Independent variables	Actual values	Coded values
Solvent concentration (%)	40	-1
	70	0
	100	+1
Time (min)	5	-1
	10	0
	15	+1
Solid-to-solvent ratio (mg/ml)	25	-1
	50	0
	75	+1

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701 **Table 2.** Nutritional composition of *S. cinereum*

Components (%)	Dry weight basis	Wet weight basis
Moisture	-	80.20 ± 0.19
Ash	12.52±0.46	2.48 ± 0.09
Crude protein	13.35±0.41	2.64 ± 0.07
Crude lipid	00.97±0.02	0.19 ± 0.005
Crude fiber	20.00±0.47	3.96 ± 0.13
Carbohydrate	53.15 ± 0.28	10.52 ± 0.04
Energy (Kcal/100 g)	274.73	54.35

702 All the values represent the means with the standard deviation of triplicate.

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720 **Table 3.** Mineral composition of the brown macroalgae *S. cinereum.*

Components	Quantity (mg/g DW)
Macrominerals	
Nitrogen	22.10±0.13
Phosphorous	0.239±0.01
Potassium	15.26±0.14
Sodium	11.46±0.05
Sulphur	11.69±0.11
Calcium	40.00±0.25
Magnesium	16.50±0.06
Microminerals	
Boron	0.48±0.00
Zinc	3.41±0.00
Iron	9.71±0.00
Manganese	7.88±0.00
Copper	5.81±0.00

721 All the values represent the means with the standard deviation of triplicate.

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735 **Table 4.** Fatty acid composition of *S. cinereum*

Fatty Acid name	Formula	Retention time	Area %
Myristic acid	C14:0	16.035	6.512
Palmitic acid	C16:0	19.366	48.012
Palmitoleic acid	C16:1	19.689	5.694
Stearic acid	C18:0	22.442	4.267
Oleic acid	C18:1n	22.657	17.381
Linoleic acid	C18:2	23.263	6.006
α-Linolenic acid	C18:3n6	24.117	4.295
Cis-11-Eicosaenoic acid	C20:1	25.544	1.507
Arachidonic acid	C20:4n6	26.657	6.327
Total			**100**

Table 5. RSM-BBD experimental design for optimization of UAE parameters for the extraction of bioactives from *S. cinereum* with independent variables and their respective responses

Run	Independent variables (X)			Responses (Y)					
	Solvent concentration % (X_1)	Time min (X_2)	Solid:solvent (mg/ml) (X_3)	TPC (mg/g GAE) (Y_1)		TFC (mg/g QE) (Y_2)		DPPH Inhibition (%) (Y_3)	
				Actual	Predicted	Actual	Predicted	Actual	Predicted
1	70	15	75	0.3380	0.4626	2.01	1.94	6.57	6.10
2	70	5	25	0.4641	0.6583	2.04	2.84	1.64	2.11
3	40	10	75	0.8327	0.7724	0.9010	1.36	18.51	18.58
4	70	5	75	0.1776	0.3633	0.9102	1.14	5.02	5.54
5	100	10	75	0.1892	0.0535	1.74	1.72	4.44	4.31
6	40	15	50	0.8345	0.9695	2.60	2.61	13.66	14.06
7	100	15	50	0.1649	0.2507	2.21	2.98	5.89	6.49
8	100	10	25	0.3125	0.3485	4.10	3.43	5.46	5.39
9	40	5	50	0.9157	0.8702	2.18	1.81	16.28	15.68
10	70	10	50	0.6693	0.5604	3.14	2.39	8.65	9.24
11	40	10	25	1.14	1.07	2.81	3.06	10.34	10.46
12	70	10	50	0.5736	0.5604	1.54	2.39	9.18	9.24
13	70	15	25	0.7994	0.7576	3.43	3.65	3.02	2.50
14	70	10	50	0.6983	0.5604	3.13	2.39	7.76	9.24
15	70	10	50	0.5359	0.5604	2.88	2.39	9.45	9.24
16	100	5	50	0.1823	0.1514	1.91	2.17	4.30	3.91
17	70	10	50	0.6983	0.6676	3.13	2.39	11.14	9.24

Table 6. ANOVA and Regression Coefficients of second-order polynomial models of RSM-BBD optimization experiment**.**

Type of effects	Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Regression Coefficients
TPC (Linear model)							
Linear	Model	1.23	3	0.4091	26.93	<0.0001	**Intercept** 0.5604
	X_1	1.03	1	1.03	68.02	<0.0001	-0.3594
	X_2	0.0197	1	0.0197	1.30	0.2752	0.0496
	X_3	0.1741	1	0.1741	11.46	0.0049	-0.1475
	Residual	0.1975	13	0.0152			
	Lack of Fit	0.1748	9	0.0194	3.41	0.1247	
	Pure Error	0.0228	4	0.0057			
	Cor Total	1.42	16				
TFC (Linear model)							
Linear	Model	7.39	3	2.46	6.59	0.0060	**Intercept** 2.39
	X_1	0.2683	1	0.2683	0.7183	0.4120	0.1831
	X_2	1.29	1	1.29	3.46	0.0855	0.4021
	X_3	5.82	1	5.82	15.59	0.0017	-0.8533
	Residual	4.86	13	0.3735			
	Lack of Fit	2.95	9	0.3278	0.6881	0.7062	
	Pure Error	1.91	4	0.4764			
	Cor Total	12.24	16				
DPPH inhibition % (Quadratic)							
Linear	Model	333.96	9	37.11	31.70	<0.0001	**Intercept** 9.24
	X_1	187.17	1	187.17	159.89	<0.0001	-4.84
	X_2	0.4538	1	0.4538	0.3877	0.5533	0.2382
	X_3	24.77	1	24.77	21.16	0.0025	1.76
Interaction	X_1X_2	4.41	1	4.41	3.77	0.0933	1.05
	X_1X_3	21.13	1	21.13	18.05	0.0038	-2.30
	X_2X_3	0.0076	1	0.0076	0.0065	0.9382	0.0435
Quadratic	X_1^2	43.42	1	43.42	37.09	0.0005	3.21
	X_2^2	24.53	1	24.53	20.96	0.0026	-2.41
	X_3^2	32.14	1	32.14	27.46	0.0012	-2.76
	Residual	8.19	7	1.17			
	Lack of Fit	2.03	3	0.6760	0.4385	0.7379	
	Pure Error	6.17	4	1.54			
	Cor Total	342.15	16				

Table 7. Fit statistics for the RSM-BBD model for the optimization process for extracting bioactives from *S. cinereum*

Responses	**TPC**	**TFC**	**DPPH inhibition %**
R^2	0.8614	0.6033	0.9761
R^2_{adj}	0.8294	0.5118	0.9453
R^2_{pred}	0.7441	0.3741	0.8770
Adeq Precision	16.9568	8.4689	19.8536
CV%	21.99	25.55	13.02
Model strength	Moderate to Good	Moderate	Strong

Table 8. Optimized parameters of ultrasound-assisted extraction for bioactive compounds from *S. cinereum*

Independent variables	Optimum parameters		Responses					
			(Y_1) TPC (mg/g GAE)		(Y_2) TFC (mg/g QE)		(Y_3) DPPH inhibition (%)	
	Coded levels	Actual levels	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
Solvent concentration (X_1)	-1	40.0	1.09495	1.0234	2.6289	2.86115	12.0711	13.3404
Time (X_2)	0.464	12.32						
Solid-to-solvent ratio (X_3)	-0.546	36.35						

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Figure 1. Habitat and collection of *Sargassum cinereum* from the rocky shores of Uttara Kannada district, Karnataka, India. (A) Panoramic view of the Bhatkal coastline, (B) High standing-stock population of *S. cinereum* attached to a rocky intertidal surface, (C) Bulk biomass collected for subsequent processing and analysis, and (D) Representative thallus morphology of *S. cinereum* used for taxonomic identification.

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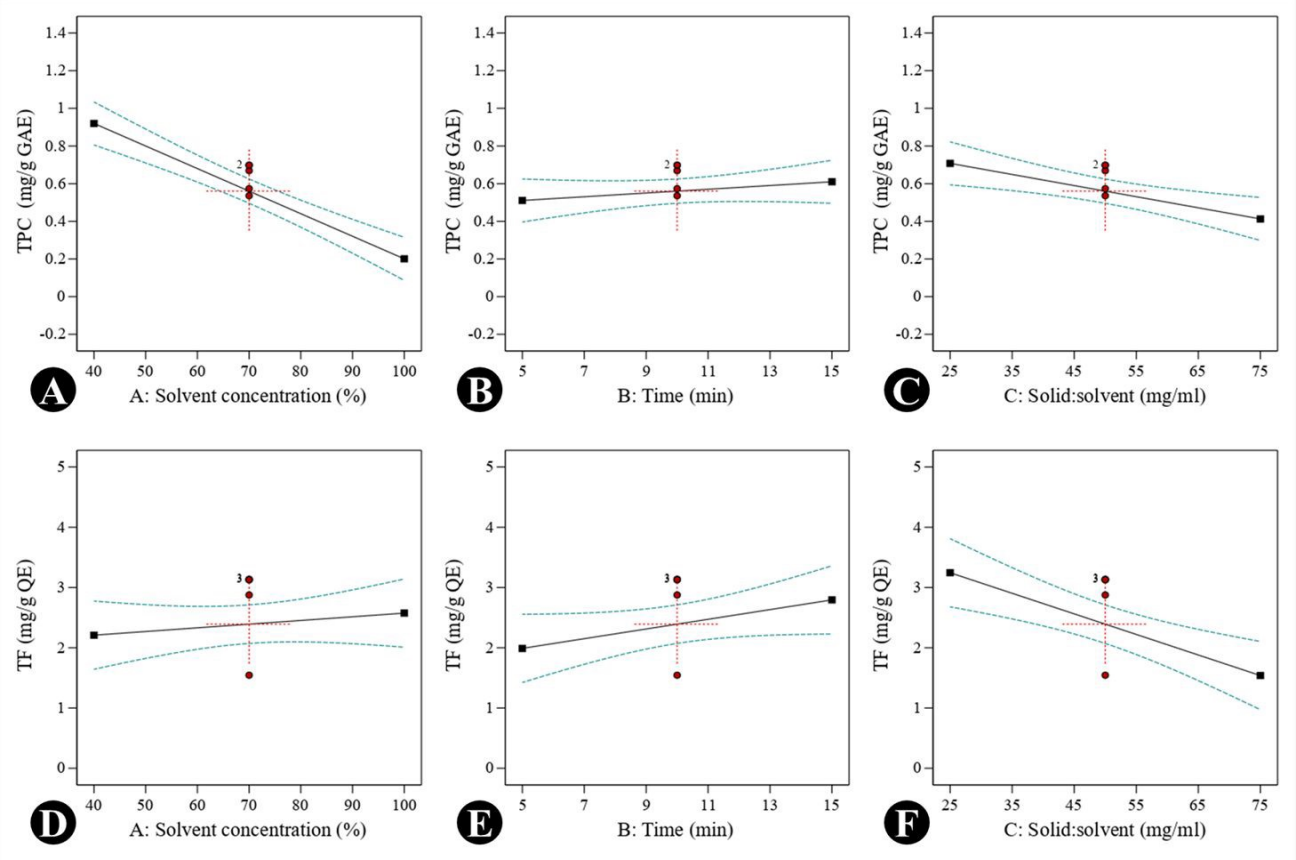

Figure 2. RSM model graphs of UAE optimization for bioactives from *S. cinereum*, showing the effect of individual independent variables X_1, X_2, and X_3 on response values (A-C) TPC and (D-F) TFC

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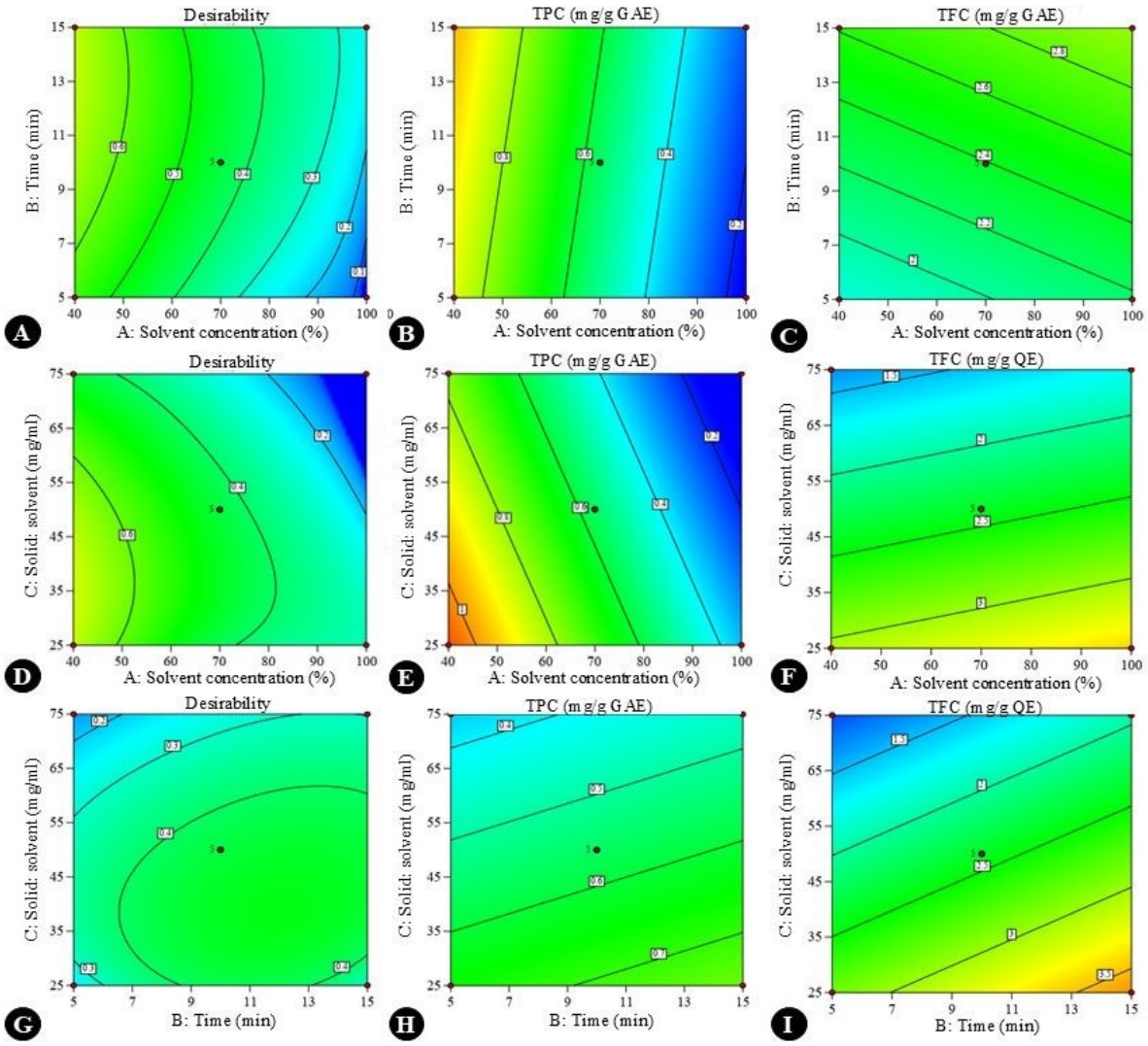

Figure 3. Contour graphs showing the effect of interaction among the variables on response values TPC and TFC in the extraction optimization process along with desirability. Effect of (A-C) interaction between solvent concentration and time, (D-F) interaction between solvent concentration and solid to solvent ratio, and (G-I) interaction between time and solid to solvent ratio (In each interaction plot, the third independent variable was maintained at its central level as specified in Table 1)

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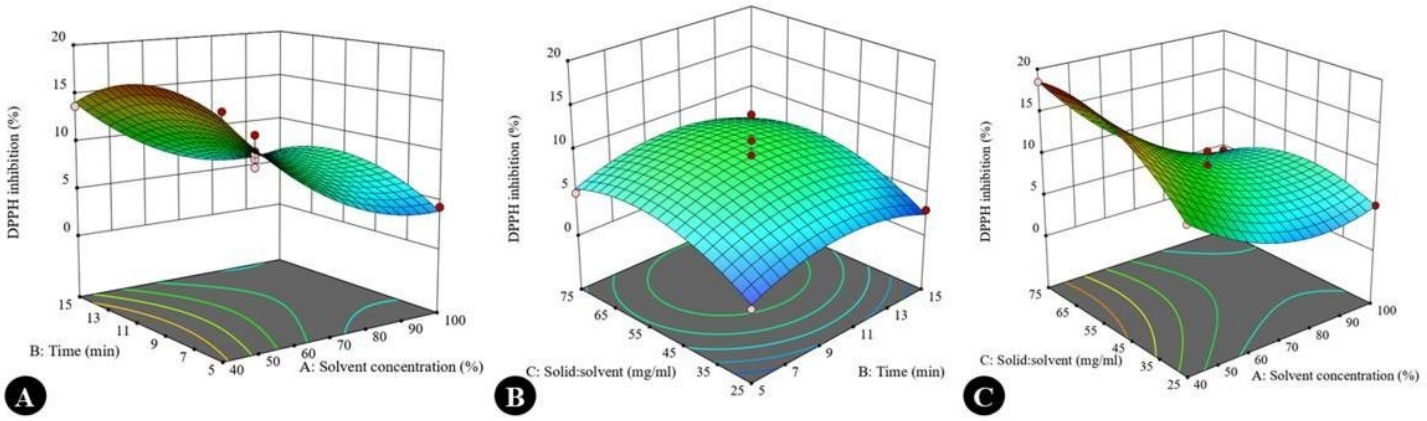

Figure 4. 3D response graphs showing the effect of interaction between variables on DPPH inhibition % of *S. cinereum* extract: (A) solvent concentration and time, (B) time and solid to solvent ratio, and (C) solvent concentration and solid to solvent ratio (In each interaction plot, the third independent variable was maintained at its central level as specified in Table 1)

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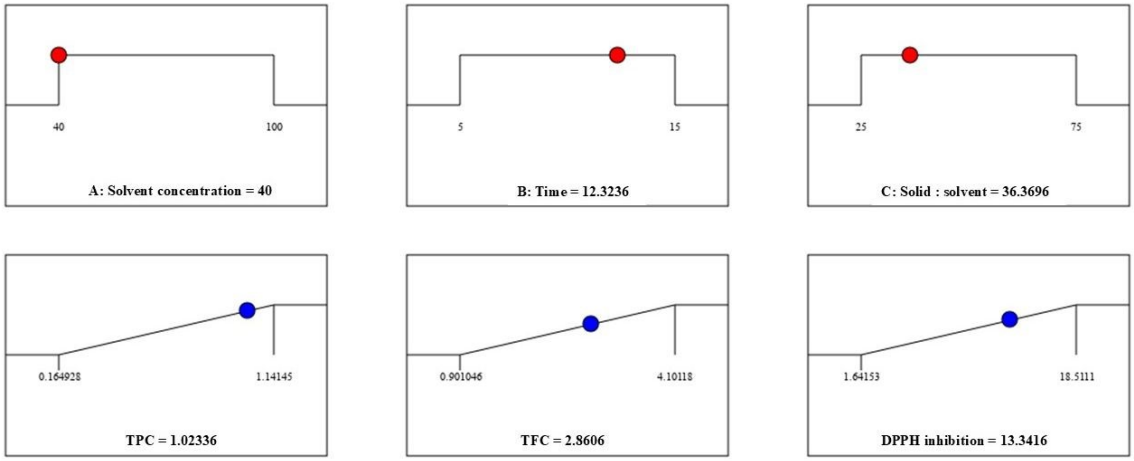

Figure 5. Optimization desirability solution graph from RSM-BBD for the UAE of bioactives from *S. cinereum*

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Data availability statement

Any other external data are not used for this manuscript; all the data are generated from experimental analysis.