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Fucoxanthin Content and Antioxidant Activity in Supercritical CO_2, Enzymatic and Natural Hydrophobic deep Eutectic Solvent Extracts of *Sargassum wightii* Seaweed

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Abstract

Brown seaweeds are potential sources of bioactive compounds which can be beneficial in the development of novel nutraceuticals and cosmeceuticals. Green chemistry techniques, namely supercritical fluid (SFE), enzymatic and natural deep eutectic solvent (NaDES) extraction, were employed on *Sargassum wightii* seaweed. LC-MS/MS estimation revealed that the SFE technique could extract the maximum amount of fucoxanthin (3.00±0.04 mg g^{-1} seaweed), followed by NaDES (1.7±0.006 mg g^{-1} seaweed) and enzymatic extraction (0.17±0.003 mg g^{-1} seaweed). However, total phenolic content, expressed as mg gallic acid equivalent g^{-1} extract, was highest in enzymatic (90±2.6), followed by SFE (61.02 ±1.4) and NaHDES (55.35± 2.06) extracts. The DPPH free radical scavenging activity was highest in the SFE extract (IC_{50} 2.07±0.04 μg gallic acid equivalent). There was no significant difference in the DPPH free radical scavenging activity between the enzymatic (IC_{50} 13±3.24 μg gallic acid equivalent) and NaDES extracts (IC_{50} 16.64±2.04 μg gallic acid equivalent). Interestingly, ABTS free radical scavenging activity was highest in the enzymatic extract (IC_{50} 11±2.06 μg Trolox equivalent), followed by SFE and NaDES extracts. The study reveals that all the three methods are excellent alternatives to harmful organic solvents. The result indicates that SFE is the best method among all the employed techniques.

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Introduction

The seaweed genus Sargassum is a well-known source of several unique phytochemicals, including anti-obesogenic and anti-diabetic carotenoid fucoxanthin and antioxidant polyphenols. Such promising bioactivities has led researchers to screen these phytochemicals in several Sargassum species (Qiu et al., 2021). Almost 56 Sargassum species grow abundantly in the Indian coastline, of which *Sargassum wightii* is the most predominant and commercially exploited species. However, recently, researchers reported fucoxanthin's isolation from an ethyl acetate extract of *Sargassum wightii* (Raji et al., 2020). Moreover, various works have focused on studying the efficacy of dietary fucoxanthin and its effective use as a dietary supplement (Wang et al., 2018).

Novel extraction techniques and green solvents are increasingly promoted in the extraction of nutraceuticals from seaweed (Ummat et al., 2021). Such methods often improve extraction efficiency and reduce the environmental footprint of the process. Particularly, extraction using supercritical CO_2, enzymes, and natural deep eutectic solvents holds immense potential (Miyashita et al., 2020). Supercritical fluid CO_2 extraction (SFE) of fucoxanthin from *Sargassum muticum*, *Sargassum binderi* and *Sargassum horneri* has been reported earlier (Conde et al., 2015; Balboa et al., 2015; Jaswir et al., 2017; Sivagnanam et al., 2015). Enzyme assisted extraction techniques have been employed for the extraction of

seaweed polyphenols and fucoxanthin (Ummat et al., 2021, Billakanti et al., 2013). Natural Deep Eutectic solvents (NaDES) are emerging class of inexpensive green solvents with low to no cytotoxicity, finding promising applications in the extraction of flavonoids, phenolic acids, polyphenols, tanshinones, rosiglitazones, saponins, anthraquinones and carrageenan from various types of natural sources (Zainal-Abidin et al., 2017). In the available literature, these green technologies have not been applied for the extraction of fucoxanthin and phenolics from *Sargassum wightii*. This study aims to provide a comparative account of fucoxanthin content, total phenolic content and *in vitro* antioxidant activity in extracts of *Sargassum wightii* obtained by SFE, enzymatic and NaDES extraction.

Materials and Methods

Brown seaweed *S. wightii* was collected from the coastline of Mandapam region of Tamil Nadu (9°172 N and 79°112 E), India. The collected samples were washed thoroughly with tap water to remove shells and sand particles and dried under solar drier at 45°C for 12 h. The dried samples were pulverizsed and stored under refrigerated condition (2-8°C) in Zip lock bags for further analyses. Analytical standard of fucoxanthin, HPLC grade methanol and tert-Butyl methyl ether (MTBE); Gallic acid, DPPH, ABTS and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Papain, Viscozyme®, sodium acetate, sodium phosphate, ascorbic acid, and folin-ciocalteu reagent were procured from Merck (Mumbai, India).

Three different green extraction techniques such as supercritical fluid extraction (SFE), deep eutectic solvent extraction (Natural deep eutectic solvent-NaDES and hydrophobic deep eutectic solvent-HDES extraction) and enzymatic extraction (EE) were conducted. All the extracts were subjected to antioxidant and fucoxanthin quantification to analyze and compare the solvent extraction efficiency. In SFE, the supercritical CO_2 was used as the solvent with ethanol as modifier. The best extraction mode was previously optimized in terms of pressure, time and amount of modifier (400 bar pressure, 3 h time and 15% co-solvent). The extract was evaporated off the solvent and stored under refrigeration (2-4°C) until further use.

For NaDES and HDES extraction, the first step was the development of solvent system using both hydrogen bond donors and acceptors. In this study, 11 different deep eutectic solvents were synthesized, namely, Choline chloride + Urea (CC+U 2:1); Choline chloride + Malic acid (CC+ M.a 1:1) (60-70°C); Choline chloride + Glycerol (CC+Gly 1:2); Choline chloride + Lactic acid (CC+L.a 1:1); Choline chloride + ethylene glycol (CC+ Eg 1:2); Fructose + Citric acid (Frt+ C.a 1:3) (60-70°C); Choline chloride + Glycerol + Water (CC+Gly+H2O 5:1:5); Malic acid + Fructose + Glycerol (M.a+ Frt+ Gly 3:1:3); Lactic acid + Dextrose + Water (L.a+Dxt+H2O 6:1:6); Malic acid + Dextrose + Water (M.a+Dxt+H2O 3:2:3); and Menthol + Lactic acid (Me+La.a 1:2) (HDES). The synthesis protocols were optimized using different molar ratio and reaction temperatures. For extraction, an amount of 2 g powdered seaweed in 20 ml solvent was mixed thoroughly using magnetic stirrer for 5 h. The seaweed particle from the extract was removed by passing through a muslin cloth followed by centrifugation (4000 rpm for 20 min). The clear extract was stored under refrigeration until further use. The enzymatic extraction of seaweed followed protocols reported in literature (Habeebullah et al., 2021)

A LC-MS/MS (Sciex 6500+) in multiple reaction monitoring (MRM) mode determined the fucoxanthin content in each extract. The optimized MRM conditions are presented in Table 1. A Phenomenex Kinetex® column (2.6 μm C18 100Å, 150 × 4.6 mm) offered satisfactory chromatography of Fucoxanthin, using a gradient program of seven-minute with water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B) at a flow rate of 0.5 mL/min. The column oven maintained a temperature of 45ºC throughout the analyses. Further, MS/MS spectra of absolute fucoxanthin solvent standard were acquired and compared with MS/MS spectra of fucoxanthin in the SFE fractions for confirmation of identity (Fig. 1).

The phenolic concentration was analyzed by Folin-Ciocalteu reagent procedure (Arulkumar et al., 2018) with minor modifications. Standard gallic acid, in the concentration range of 20 to 100 μg/ml was used for calibration. The phenolic content of the sample solutions were expressed as gallic acid equivalent per gram (GAE/g) extract.

DPPH radical quenching assay was performed as described in the previously reported research work (Blois et al., 1958). The IC_{50} was represented as mg Gallic Acid Equivalent (GAE) per gram extract. The

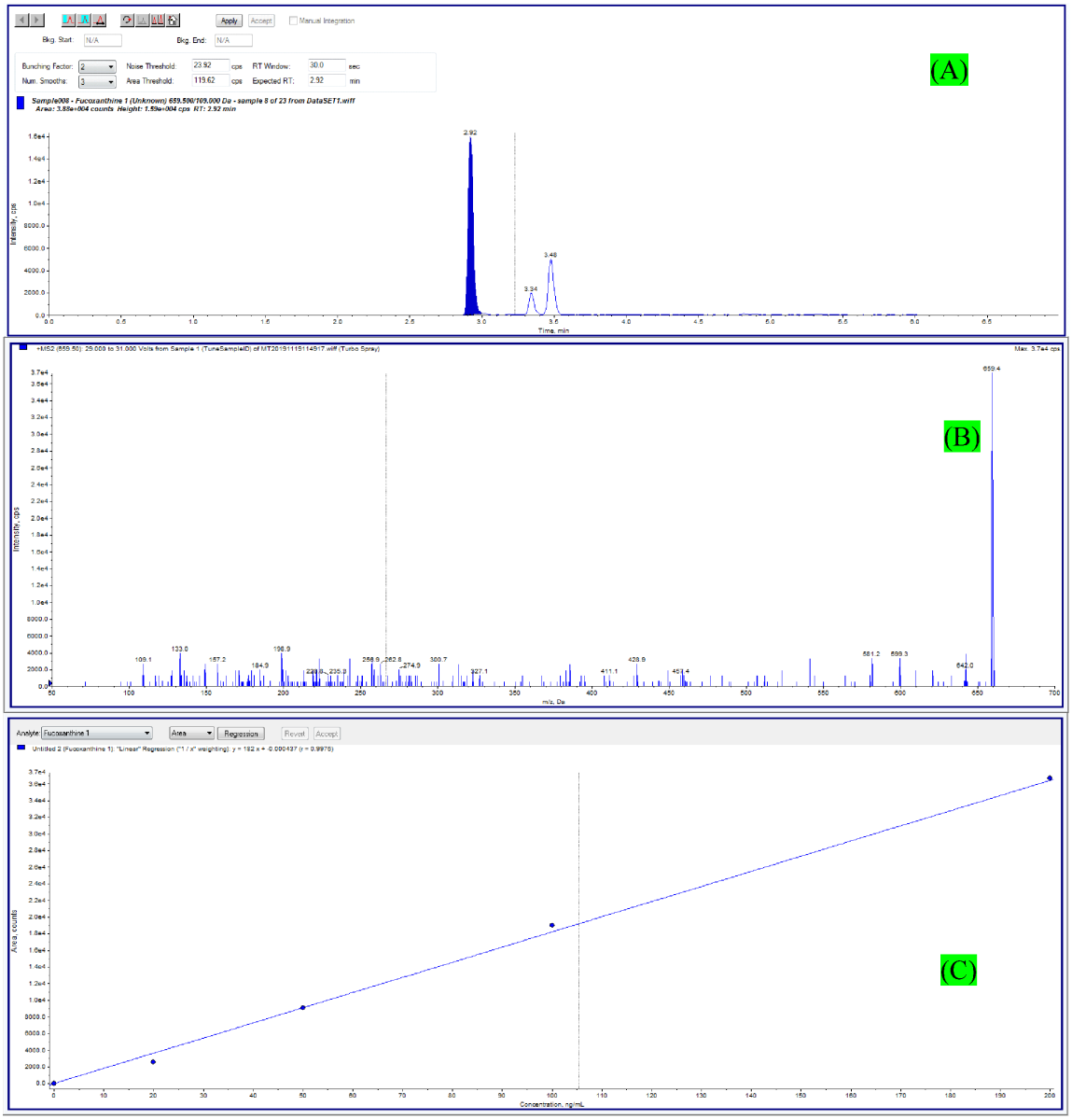

Fig. 1A. Representative quantifier ion peak of Fucoxanthin in *Sargassum wightii* extract; B. MS/MS spectra of fucoxanthin in extract showing characteristic parent ion and product ions; C. Representative calibration curve for quantification of fucoxanthin

percentage of inhibition was determined by applying the equation,

$$\% \ Inhibition = (A_0 - A_1)/A_0 \qquad (1)$$

Where A_0 = Absorbance of the standard; A^1 = Absorbance of sample

The ABTS free radical scavenging potential of the samples were analyzed as previously reported with minor modifications (Chan et al., 2012). The IC_{50} value of each extract against ABTS radicals was expressed as µg Trolox equivalent (TE) per gram extract. The percentage of inhibition was calculated as in equation (1).

Result and Discussion

Confirmatory analysis of fucoxanthin content in the extracts was carried out using LC-MS/MS in MRM mode, comparing against an external analytical standard of fucoxanthin. Characteristics precursor ion and two product ions unequivocally confirmed

Table 1. Optimised multiple reaction monitoring (MRM) parameters of fucoxanthin for quantification and confirmation by LC-MS/MS

Q1 Mass (Da)	Q3 Mass (Da)	Duel Time (msec)	Declustering potential (DP)	Entrance potential (EP)	Collision Energy (CE)	Collision cell exit potential (CXP)
659.5	109	100	48	10	28	18
659.5	581.4	100	48	10	28	18

the presence of fucoxanthin. Supplementary material to this manuscript presents the MS/MS spectra, optimized MRM parameters and the representative peak of fucoxanthin in a sample. The SFE extraction parameters were optimized using a Box-Behnken design of experiment, which suggested 400 bar pressure, 15% ethanol as co-solvent and 3 h of extraction as optimum. The optimized SFE technique could extract 3.00 ± 0.04 mg fucoxanthin per gram of dry *Sargassum wightii* powder. Seven different NaDES were evaluated for extraction, namely Menthol-Lactic acid (Me+L.a 1:2), Lactic acid-Dextrose-Water (L.a+Dxt+H_2O 6:1:6), Malic acid-Fructose-Glycerol (M.a+Frt+Gly 3:1:3), Malic acid-Dextrose-Water (M.a+Dxt+H_2O 3:2:3), Choline chloride-Glycerol-Water (C.C+Gly+H_2O 5:1:5), Fructose-Citric acid (Frt+C.a 1:3) and Choline chloride-Lactic acid (C.C+L.a 1:1). The hydrophobic Me+L.a NaDES could extract 1.7±0.006 mg fucoxanthin from a gram of dry *Sargassum wightii* powder. Fucoxanthin was not present in any other NaDES extracts. Sequential enzymatic extraction of the dry seaweed powder with Viscozyme® and Protamex® extracted 0.17±0.003 mg fucoxanthin from a gram. When compared with other Sargassum species, the fucoxanthin content in *Sargassum wightii* was better or equivalent. Earlier reports suggest that the SFE technique could extract 0.77 to 5 mg fucoxanthin from a gram of dry Sargassum powder, depending on the species and season (Miyashita et al., 2020).

Total phenolic content was higher in the enzymatic extracts (90±2.63 mg gallic acid equivalent(GAE)/g) when compared to SFE and NaDES extracts. The SFE extracts contained 61.02 ±1.4 mgGAE/g, while the phenolic content in the NaDES extracts ranged from 49.62±0.62 to 78±2.6 mgGAE g^{-1}. Use of ethanol as polarity modifier, along with supercritical CO_2 might have helped in extraction of phenolic acids. The nonpolar NaDES i.e. Me+L.a (1:2) extract contained 55.35± 2.06 mgGAE g^{-1} total phenolics which was lesser than more polar NaDES (Fig. 2. A).

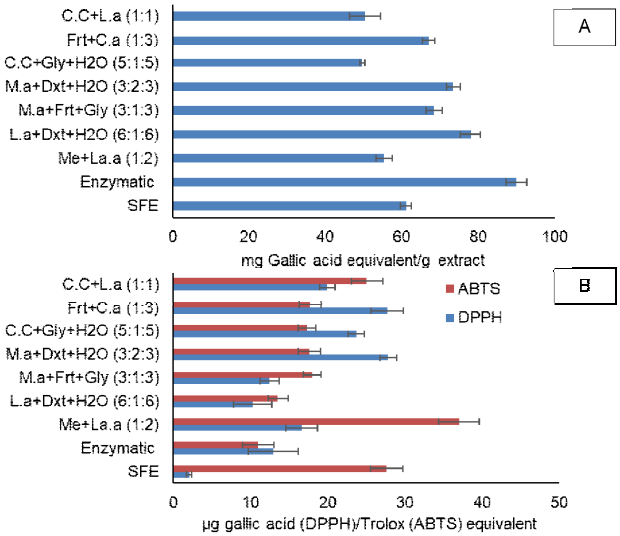

Fig. 2A. Total phenolic content in different extracts; B. IC_{50} values of different extracts for DPPH and ABTS free radical scavenging activity

The SFE extract exhibited a significant free radical reducing potential with IC_{50} values of 27.62±2.06 µg Trolox and 2.07±0.04 µg Gallic acid equivalent for ABTS and DPPH respectively. Eventhough the DPPH free radical scavenging activity was the best in SFE extract, the enzymatic extract demonstrated better ABTS radical scavenging activity. Overall the enzymatic extract was best among all the extract in terms of free radical scavenging activity (Fig. 2B.). It was interesting to note that the SFE extract showed the best DPPH free radical scavenging activity. However, the ABTS free radical scavenging activity was comparatively inferior. Better DPPH activity of the SFE extact could be due to the presence of phlorotannins. Previous report reveals that phlorotannins are bi-polar phenolic compounds and exhibit high radical scavenging potential for DPPH but lesser for ABTS (Archana & Vijayalakshmi, 2018).

Sargassum wightii from Indian coastline could be a valuable source of nutraceutical molecules such as fucoxanthin. Fucoxanthin content in the seaweed was similar or superior to other Sargassum sp., screened previously by other researchers. Among the green extraction techniques SFE was most promising for fucoxanthin extraction. However, the phenolic acid content and free radical scavenging activity were superior in the enzymatic extract. The extracts using NaDES contained considerable amount of phenolics, and demonstrated radical scavenging activity. The results reiterate the potential of green extraction techniques in developing nutraceutical products from seaweed resources.

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