Comparison of Phytochemicals and Antioxidant Activity of the Polysaccharide and de-polysaccharide Methanol Extracts of Brown Seaweed Chnoospora minima

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Abstract**:** *Chnoospora minima is a brown alga enriched with unique bioactive compounds which are ideal candidates for pharmaceutical, cosmeceutical and nutraceutical industries. During this study, C. minima was extracted using 80% methanol and de-polysaccharide crude methanol extract was obtained by ethanol precipitation followed by separation of the polysaccharide portion. The crude methanol extract of C. minima and its fractions were subjected to analyse phytochemicals and antioxidant activities.*

For the assessment of radical scavenging activity, DPPH, FRAP, ABTS$^{+,}$ and ORAC assays were conducted. Ethyl acetate fractions of both polysaccharide (IC_{50}:0.67 ± 0.01 mg/mL) and de-polysaccharide (IC_{50}:0.59 ± 0.015 mg/mL) crude methanol extracts exhibited DPPH radical scavenging activity in terms of antioxidant activity. Similarly, the highest level of ORAC, FRAP, and ABTS+ activity was observed in the ethyl acetate fractions of de-polysaccharide (ORAC:19.73 ± 5.31 mg TE/g; FRAP: 20.34 ± 1.72 mg TE/g; ABTS+: 0.06 ± 0001 IC50(mg/ml)) and polysaccharide crude methanol extracts (ORAC:16.22 ± 4.31 mg TE/g; FRAP: 19.23 ± 1.98 mg TE/g; ABTS^{+}:0.08 ± 0.002 IC50(mg/ml)). High TPC was observed in the depolysaccharide crude methanol extract (298.07 ± 0.003 mg GAE/g) and aqueous fraction (141.2 ± 0.002 mg GAE/g) of the polysaccharide crude methanol extract. Highest TFC was observed in both aqueous fractions of depolysaccharide (594.23 ± 0.001 mg QE/g) and polysaccharide (113.46 ± 0.001 mg QE/g) crude methanol extracts. Chloroform fractions exhibited the highest TAC for polysaccharide (2.20 ± 0.45 mg PE/g), and depolysaccharide (2.79 ± 0.31 mg PE/g) samples. Therefore, the depolysaccharide C. minima sample exhibited high level of antioxidant activity along with a high content of phytochemicals which can further be utilized to determine bioactivities that lead to future drug development.

Keywords: *Chnoospora* minima, de-polysaccharide extract: polysaccharide extract: Antioxidant activity, Phytochemicals

1. Introduction

Marine biotechnology is a broad field where demand for new research and development techniques is rise up day by day. When considering pharmaceutical, nutraceutical, and cosmeceutical industries, marine bioresources including macro and microalgae, crustaceans, marine mammals and seaweeds play a vital role in it (Wan-Loy and Siew-Moi, 2016) (Torres et al., 2019).

Sri Lanka is a tropical country located in the Indian ocean near to the equator with about 29°C of average temperature in its coastal areas. Due to this favourable climate conditions available in the coastal areas, it provide the habitat for lots of marine biosources including algae and acts as a hub for different marine plant varieties to grow (Fernando, 2017). Among those marine bioresources, marine algae are ideal candidates with vast variety of biologically active compounds including terpenoids, alkaloids, polyphenols, sulphated polysaccharides, peptides, amino acids, halogenated compounds and polyunsaturated fatty acids that are responsible for antioxidant, anti-cancer, anti-diabetic, anti-wrinkle, anti-aging, anti-inflammatory, anti-obesity and anti-bacterial activities (Dyshlovoy and Honecker, 2015) (Dobretsov et al., 2016). In general, algae can be classified in to three main segments as rhodophyta, chlorophyta, and phaeophyta based on their colour which is occurred as a result of the availability of photosynthetic pigments (Çakir Arica et al., 2017) (Abirami and Kowsalya, 2016). Out of these important marine algae, Chnoospora minima is a marine alga that can be found near the coastal areas of Mannar, Hikkaduwa, Galle, and Nilaveli in Sri Lanka.

Most commonly, detection of phytochemicals represents the bioavailability of polyphenols, flavonoids and alkaloids within natural extracts which are encountered for different medicinal properties including anti-cancer, antidiabetic, anti-inflammatory and anti-bacterial etc (Çakir Arica et al., 2017). Similarly, investigation of anti-oxidant activities centered with the formulation of novel food and drugs as they are totally deal with the human health and wellbeing to mitigate different diseases or to counteract oxidative stress (van Weelden et al., 2019) (Gutiérrez-Rodríguez et al., 2018). Alternatively, these antioxidants can eliminate or are able to reduce lipid peroxidation which in turn responsible for the prolonging of food and drug shelf lives. In addition to that, these antioxidant activities are ideal for skin treatments as well (van Weelden et al., 2019) (Ganesan, Kumar and Bhaskar, 2008).

In this investigation, it intended to explore, compare and contrast the phytochemical composition and antioxidant activities of polysaccharide and de-polysaccharide samples and fractions of Sri Lankan marine alga Chnoospora minima, and to investigate best sample out of them in order to examine their potent incorporation in pharmaceutical, cosmeceutical and nutraceutical industry applications.

2. Methodology

1) Sample preparation

1.1) Polysaccharide rich methanol extract: Previously collected and freeze-dried C. minima alga samples were taken and finely powdered using mechanical grinder. Then 200g of powdered C. minima samples were extracted to 80% methanol (5L) using sonication method for 90 minutes at 25 °C (3 times). Methanol extracts of C. minima were then filtered for three times using ceilite bed packed in a sinter funnel and concentrated by using a rotary evaporator at

48 °C. Concentrated polysaccharide crude methanol extract of C. minima was then freeze dried to obtain solid crude methanol extract to remove all water and obtained solid crude methanol extract was stored at -20 °C until further use. Obtained solid crude methanol extract were dissolved in DMSO to prepare known stock sample concentrations. (10g/ml) (Gunathilaka et al., 2019).

1.2) De-polysaccharide crude methanol extract: Previously collected, freeze dried C. minima algae samples were taken and finely powdered using mechanical grinder. 200g of powdered C. minima samples were extracted to 80% methanol (5L) using sonication method for 90 minutes at 25 °C (3 times). Methanol extracts of C. minima were then filtered for three times using ceilite bed packed in a sinter funnel and concentrated by using a rotary evaporator at 48 °C. polysaccharide crude methanol extract of C. minima(concentrated) was precipitated overnight with ethanol to remove all the polysaccharides. Thereafter, to remove the ethanol content, samples were subjected to concentration via rotary evaporator. Concentrated de-polysaccharide C. minima crude methanol extract was then freeze dried to obtain solid crude methanol extract and obtained solid crude methanol extract was stored at -20 °C until further use. Obtained solid crude methanol extracts were dissolved in DMSO to prepare known stock sample concentrations. (10g/ml) (Gunathilaka et al., 2019).

2) Solvent fraction preparation

250mg of both powdered polysaccharide and de-polysaccharide crude methanol extracts were dissolved in 100 ml of deionized water separately and partitioned. Partitioning was done using 100ml hexane, chloroform and ethyl acetate respectively according to the ascending order of their polarity (100 ml X 3 times) to obtain four fractions from samples as Hexane, chloroform, ethyl acetate and aqueous fractions. After the partitioning process, all fractions were evaporated for overnight to remove all the solvents (air dry). All dried fractions were stored at -20 °C until use. Finally, all fractions were dissolved in DMSO to make known concentrations of stock samples. (10mg/ml).

3) In vitro phytochemical assays

3.1) Total polyphenolic content (TPC) determination: Total polyphenolic content of both polysaccharide and de-polysaccharide crude methanolic extracts and all fractions of C. minima was determined using Folin-Ciocalteu reagent. In here, 20 µL of crude alga extracts and hexane, chloroform, ethyl acetate and aqueous fractions were mixed with freshly prepared Folin-Ciocalteu reagent (110 µL of 10 times diluted) freshly prepared and added 70 µl of sodium carbonate solution to neutralize the solution. Then, the 96 well plate (fully covered with aluminium foil) with the solution was incubated at room temperature for 30 min and its absorbance was measured using spectrophotometer at the wavelength of 765nm by using water as the sample blank (Gunathilaka et al., 2019).

3.2) Total Flavonoid content (TFC) determination: Determination of the total flavonoid content of both polysaccharide and de-polysaccharide crude methanol extracts of C. minima were determined by using the aluminium chloride. In here, crude algal extracts and all the other fractions of C. minima were mixed with absolute methanol and 100 µL of the sample was mixed with 100µL of 2% $AlCl_3$ solution in methanol. Then the 96 well plate with samples were full covered with an aluminium foil and incubated at room temperature for 10 minutes and the absorbance was measured at the wavelength of 415nm using spectrophotometer (Gunathilaka et al., 2019).

3.3) Total Alkaloid content (TAC) determination: Determination of the total alkaloid content of both polysaccharide and de-polysaccharide methanol fractions of C. minima were determined by using Dragendorff reagent. 10 mg/mL algae crude methanol extracts, hexane, chloroform, ethyl acetate and aqueous fractions were diluted with 95% ethanol. Then 100 µl of the samples were mixed with 200 µL of Dragendorff reagent, and centrifuged at the speed of 5000 rpm for 5 minutes. After completing the centrifugation period, supernatant was discarded and the remaining pellet is washed with 95% ethanol and treated with 200 µL of 1% disodium sulfide solution. Then the formed pellet was again centrifuged at 5000rpm for 5 minutes. After the centrifugation, supernatant was removed and the pellet was dissolved in 200 µL of conc. HNO3 and top up with distilled water. From this solution, 100µl was taken and mixed with 500 µL of 3% thiourea inside a 15ml eppendorf tube and the absorbance was measured at a wavelength of 460 nm using spectrophotometer.

4) In vitro antioxidant assays

4.1) DPPH assay (2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay): C. minima crude methanolic extracts and all the fractions were tested with six sample concentrations ranging from 7.81µl to 125µl. In here, 40 µg/mL of DPPH was mixed with 200µl of methanol to prepare fresh DPPH solution. Then the 50 µl samples (both polysaccharide and de-polysaccharide C. minima extracts) were mixed with the DPPH solution and let for 15 minutes at room temperature for incubation. Then the absorbance was measured at 517nm wavelength and calculated the % RSA and IC50 value for every sample and the percentage inhibition was calculated using the equation of [% Inhibition= [(A control - A sample)/A control] x100] (Gunathilaka et al., 2019).

4.2) ORAC assay (Oxygen radical absorbance capacity): De-polysaccharide and polysaccharide rich C. minima crude methanolic extracts and all their fractions were tested with six sample concentrations ranging from 7.81µl to 125µl. In here, 10µl samples were added to the 96well plate and added 40 µl of phosphate buffer (pH 7.4). To it, 100µl of fluorescein was added and incubated at room temperature for 5 minutes. Then, 50 µl of AAPH solution was added to it and the decay of fluorescence was scanned for 35 minutes at 1minute interval at room temperature. Finally, the area under the curve (AUC) was recorded for the samples (Gunathilaka et al., 2019).

- ORAC value = (Net AUC sample / net AUC Trolox) * (Trolox concentration/sample concentration)
- Net AUC sample = (AUC sample – AUC blank)
- Net AUC Trolox = (AUC Trolox – AUC blank)

4.3) FRAP assay (Fluorescence recovery after photobleaching): Prepared de-polysaccharide and polysaccharide-rich C. minima extracts were subjected to determine ferric reducing antioxidant power (FRAP). In here, 150 µl of FRAP reagent was added to the 96well plate and 30 µl of acetate buffer was added to it. Then, 20µl of sample, Trolox as the standard and water as the blank was added to it and incubated at room temperature for 8minutes. Finally, the absorbance was recorded at 600nm (Gunathilaka et al., 2019).

4.3) $ABTS^{+}$ assay ((2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay: The $ABTS^{+}$ cation radical was generated by the reaction between 10mg of ABTS in 2.5mM potassium persulfate solution (2.5mL) at 37°C for 16 h in the dark. Algae fractions and sub-fractions were tested at the assay concentration range of 1-5 mg/ml. The reaction volume of 200 µL containing 40 µM of

$ABTS^+$ radical and 50 µL of extract/fraction was incubated at 25 ± 2°C for 10 minutes. The absorbance was recorded at a wavelength of 734nm. Trolox was used as the standard antioxidant, and results were expressed as mg Trolox equivalent for 1 g of the dry weight of extract/fraction. The capacity to scavenge the ABTS+ cation by 50% (IC50) was calculated from the dose-response curves by linear regression and percentage calculated using the following equation (Gunathilaka et al., 2019).

- % Inhibition= [(A control-A sample)/ A control] X 100
- A sample = absorbance of the extract/fraction
- A control = absorbance of the assay using the buffer instead of extract/fraction

Table 1. In vitro phytochemical assays (TPC, TFC, TAC) and their results for De-polysaccharideand polysaccharide crude methanol extracts of C. minima and fractions

Sample	Fraction	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg PE/g)
polysaccharide rich methanol extract	Crude	80.9±0.014	-21.15 ± 0.004	1.52±0.02
	Hexane	22 ± 0.012	-28.84 ± 0.002	1.12±0.06
	Chloroform	38 ± 0.002	78.84 ± 0.005	2.20±0.45
	Ethyl-acetate	114.2 ± 0.012	90.38 ± 0.028	1.12±0.47
	Aqueous	141.2 ± 0.002	113.46 ± 0.018	0.40± 0.03
Depolysaccharide methanol extract	Crude	298.07 ± 0.003	5.76 ±0.001	1.73±0.55
	Hexane	21.15 ± 0.002	-51.92 ± 0.001	1.36±0.69
	Chloroform	163.46 ± 0.003	-67.30 ± 0.001	2.79±0.31
	Ethyl-acetate	22.5 ± 0.006	-51.92 ± 0.001	1.43±0.47
	Aqueous	267.30 ± 0.005	594.23 ± 0.001	0.51± 0.20

3. Results and Discussions

As our results show, a high level of TPC was observed in crude methanol extract of de-polysaccharide (298.07 ± 0.003 mg GAE/g) and aqueous fraction (141.2 ± 0.002 mg GAE/g) of polysaccharide extract. The highest level of TFC was observed in both aqueous fractions of de-polysaccharide (594.23 ± 0.001 mg QE/g) and polysaccharide (113.46 ± 0.018 mg QE/g) extracts of C. minima. Chloroform fractions exhibited the highest TAC of both polysaccharide (2.20 ± 0.45 mg PE/g) and de-polysaccharide (2.79 ± 0.31 mg PE/g) extracts of C. minima.

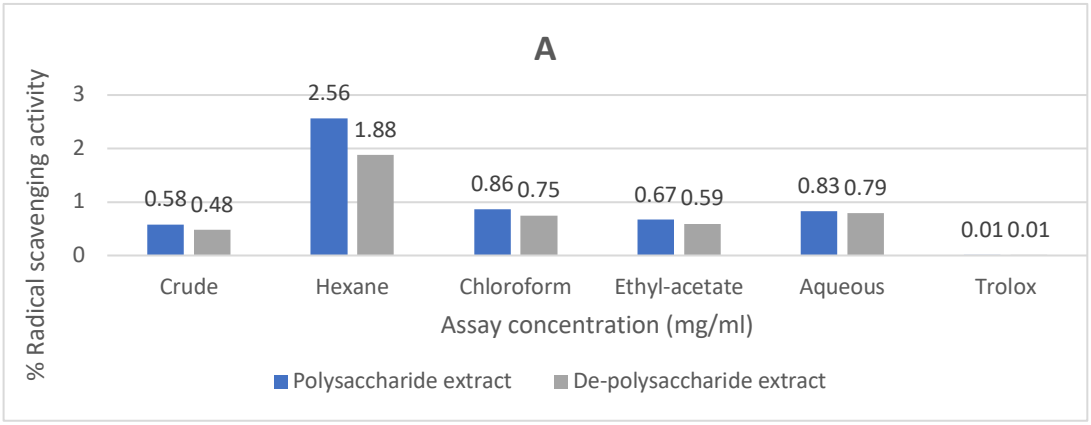
In terms of antioxidant activity determinations, we were investigated that, ethyl acetate fraction of both polysaccharide (IC50:0.67 ± 0.01 mg/ml) and de-polysaccharide (IC50:0.59 ± 0.015 mg/ml) methanol

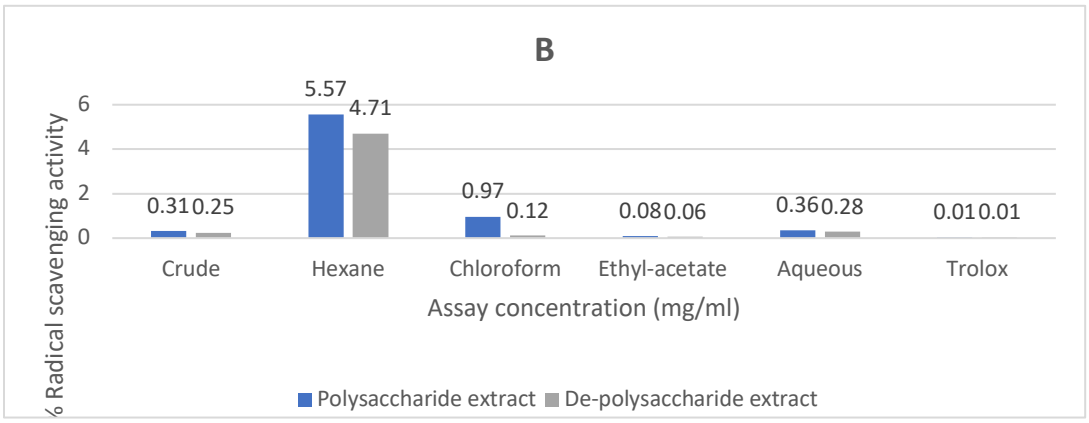
Extracts of C. minima exhibited a high level of antioxidant activity in DPPH assay. Similarly, the highest level of oxygen radical absorbance capacity was observed in the ethyl acetate fraction of both de-polysaccharide (19.73 ± 5.31 mg TE/g) and polysaccharide crude extracts of C. minima (16.22 ± 4.31 mg TE/g). Fluorescence recovery after

photobleaching (FRAP) assay shows potent antioxidant activity in ethyl acetate fraction of both polysaccharide (19.23 ± 1.98 mg TE/g) and de-polysaccharide (20.34 ± 1.72 mg TE/g) extracts of C. minima. High $ABTS^+$ was observed in ethyl acetate fractions of both polysaccharide (IC50:0.08 ± 0.002 mg/ml) and de-polysaccharide (IC50:0.06 ± 0.001mg/ml)

extracts. Based on the results it is founded that, hexane fraction of both polysaccharide and de-polysaccharide C.minima extracts are the least active

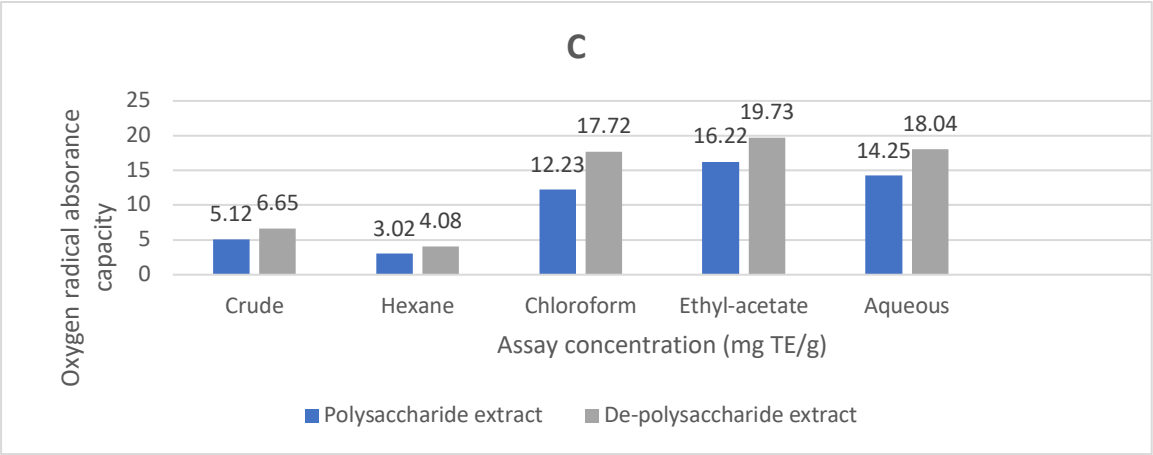
Figure 1 – Graphs for **(A)** DPPH and **(B)** ABTS+ radical scavenging activity of polysaccharide crude methanol extract, de-polysaccharide crude methanol extract and their fractions





fraction for all antioxidant assays and it can be concluded that the ethyl acetate fractions of both polysaccharide and de- polysaccharide extracts were found as ideal fractions with potential antioxidant activities and further, ethyl-acetate fraction of de-polysaccharide crude methanol extract exhibited the highest antioxidant capacity which leads to drug development.

Figure 2 – Graphs for **(C)** ORAC and **(D)** FRAP activity of polysaccharide crude methanol extract, de-polysaccharide crude methanol extract and their fractions



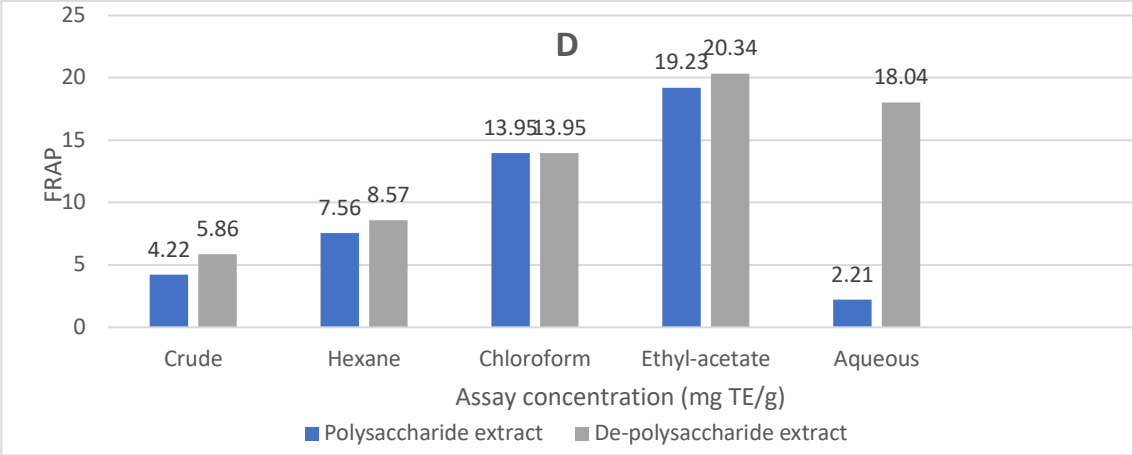


Table 2 - In vitro antioxidant assay (DPPH, ORAC, FRAP) results for De-polysaccharide and polysaccharide Crude methanol extracts of C. minima and fractions

	Fraction	DPPH IC50 (mg/ml)	ORAC (mg TE/g)	FRAP (mg TE/g)	$ABTS^{+}$ IC50(mg/ml)
C minima polysaccharide fraction	Crude	0.58 ± 0.01	5.12±0.29	4.22±1.81	0.31±0.002
	Hexane	2.56 ± 0.01	3.02±1.39	7.56±1.1	5.57±0.02
	Chloroform	0.86 ± 0.002	12.23±2.45	13.95±1.23	0.97±0.001
	Ethyl-acetate	0.67 ± 0.01	16.22±4.31	19.23±1.98	0.08±0.002
	Aqueous	0.83 ± 0.001	14.25±1.29	2.21±0.02	0.36±0.005
	Trolox	0.01 ± 0.000	-	-	0.01±0.00
C minima depolysaccharide fraction	Crude	0.48 ± 0.01	6.65±0.42	5.86±1.19	0.25±0.001
	Hexane	1.88 ± 0.02	4.08±1.44	8.57±1.13	4.71±0.31
	Chloroform	0.75 ± 0.002	17.72±2.92	13.95±1.55	0.12±0.009
	Ethyl-acetate	0.59 ± 0.015	19.73±5.31	20.34±1.72	0.06±0.001
	Aqueous	0.79 ± 0.006	18.04±1.63	4.06±0.29	0.28±0.003
	Trolox	0.01 ± 0.000	-	-	0.008±0.00

References

Abirami, R.G. and Kowsalya, S. (2016) 'Quantification and Correlation Study on Derived Phenols and Antioxidant Activity of Seaweeds from Gulf of Mannar Quantification and Correlation Study on Derived Phenols and Antioxidant Activity of Seaweeds from Gulf of Mannar', Journal of Herbs, Spices & Medicinal Plants, 00(00), pp. 1–9. doi:10.1080/10496475.2016.1240132.

Çakir Arica, Ş. et al. (2017) 'A study on the rich compounds and potential benefits of algae: A review', The Pharma Innovation Journal, 6(12), pp. 42–51. Available at: www.thepharmajournal.com.

Dobretsov, S. et al. (2016) 'Screening for anti-cancer compounds in marine organisms in Oman', Sultan Qaboos University Medical Journal, 16(2), pp. e168–e174. doi:10.18295/squmj.2016.16.02.006.

Dyshlovoy, S.A. and Honecker, F. (2015) 'Marine compounds and cancer: Where do we stand?', Marine Drugs, 13(9), pp. 5657–5665. doi:10.3390/md13095657.

Fernando, I.P.S. (2017) Bioactive natural products from marine algae and soft corals.

Ganesan, P., Kumar, C.S. and Bhaskar, N. (2008) 'Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds', 99, pp. 2717–2723. doi:10.1016/j.biortech.2007.07.005.

Gunathilaka, T.L. et al. (2019) 'In - Vitro Antioxidant , Hypoglycemic Activity , and Identification of Bioactive Compounds in Phenol - Rich Extract from the Marine Red Algae Gracilaria edulis (Gmelin) Silva', pp. 1–16. doi:10.3390/molecules24203708.

Gutiérrez-Rodríguez, A.G. et al. (2018) 'Anticancer activity of seaweeds', Drug Discovery Today, 23(2), pp. 434–447. doi:10.1016/j.drudis.2017.10.019.

Torres, P. et al. (2019) 'Review article A comprehensive review of traditional uses , bioactivity potential , and chemical diversity of the genus Gracilaria (Gracilariales , Rhodophyta)', Algal Research, 37(December 2018), pp. 288–306. doi:10.1016/j.algal.2018.12.009.

Wan-Loy, C. and Siew-Moi, P. (2016) 'Marine algae as a potential source for anti-obesity agents', Marine Drugs, 14(12), pp. 1–19. doi:10.3390/md14120222.

van Weelden, G. et al. (2019) 'Fucoidan structure and activity in relation to anti-cancer mechanisms', Marine Drugs, 17(1). doi:10.3390/md17010032.

Abbreviations

DPPH-2,2-diphenyl-1-picrylhydrazyl, TPC-Total polyphenolic content, TFC-Total flavonoid content, TAC-Total alkaloid content, FRAP-Flourescense recovery after photobleaching, ORAC-Oxygen radical absorbance capacity, C. minima- Chnoospora minima, $ABTS^{+}$ - 2,2'-Azino-Bis(3- Ethylbenzothiazoline-6-Sulphonic Acid), GAE -Gallic Acid Equivalent, ROS-Reactive Oxygen Species, TE-Trolox Equivalent.

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