



Antioxidant and Antimicrobial Potential of Hydroethanolic Extracts of *Padina tetrastromatica* from North-west Coast of India

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Abstract

Brown seaweeds are rich sources of several biologically active compounds, particularly polyphenols. The present study investigates the efficiency of hydroalcoholic solvent to extract bioactive molecules from the brown seaweed *P. tetrastromatica*. Six different combinations of ethanol in water (0, 20, 40, 60, 80 and 100% v/v) mixture were employed to extract the bioactives with an objective to study their antioxidant and antimicrobial activity. The antioxidant potential of different extracts was assessed by Total Phenolic Content (TPC), Ferric-reducing antioxidant power (FRAP) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation scavenging and Total antioxidant activity assays. 100% ethanolic extract was found to have the highest TPC and exhibited significantly higher antioxidant activity for all tests done while aqueous extract contained the highest extractive yield. The antimicrobial activity of the extracts were also evaluated against food spoilage and pathogenic bacteria using disc diffusion assay. 100% extracts significantly ($p < 0.05$) inhibited the growth of gram-negative bacteria while 60% extract also exhibited the best inhibitory effects against gram-positive bacteria. HR-LCMS analysis of the extracts revealed that 100% ethanolic extract contained a higher number of alkaloid compounds than phenolic compounds whereas aqueous extracts contained more phenolic compounds. It was observed that as the polarity decreases, the efficiency

of a hydroethanolic solvent to extract active alkaloid compounds along with polyphenols increases, which in turn contributes to the higher bioactivity of the extract.

Keywords: *Padina* sp., polyphenols, alkaloids, hydroalcoholic extract

Introduction

Seaweeds, the underutilised macro algal resources, are a rich source of phenolics and other secondary metabolites which possess a range of bioactive properties that make them potential ingredient for many industrial applications (Gupta & Abu-Ghanna, 2011). Seaweeds survive in their harsh marine environment having free radicals and strong oxidizing agents by producing protective molecules as secondary metabolites which act as reactive oxygen scavengers (Shanab et al., 2011; Mole & Sabale, 2013; Guner et al., 2015; Munir et al., 2013). Phenolic compounds, carotenoids, tannins and alkaloids with biological activity (Shahidi et al., 1992; Rice-Evans et al., 1996) attribute to the therapeutic properties of seaweeds such as anticancer, anti-obesity, antidiabetic, antihypertensive, neuroprotective and tissue healing properties *in vivo* (Mohamed et al., 2012). Therefore, seaweeds have long been used as food and medicine in many South East Asian countries including Japan, China, Thailand and Korea (Al-Enazi et al., 2018).

Among the seaweeds, brown algae are reported to have comparatively higher antioxidant potential than green and red algae (Wang et al., 2009; Cox et al., 2010; Farvin & Jacobsen, 2013; Balboa et al., 2013). *P. tetrastromatica* is one of the important brown seaweeds distributed along Indian coast, with antioxidant, antimicrobial activities and other

Received 06 March 2019; Revised 19 June 2019; Accepted 15 July 2019

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health benefits (Chatterji et al., 2004; Maheshwari et al., 2017).

The quantity and activity of bioactive compounds extracted from seaweeds largely depend on the selection of right solvent. The superiority of hydro-alcoholic mixtures among other common solvents can be explained by their polar protic nature and intermediate polarity. The alcohol's hydroxyl group can form hydrogen bonds with the oxygen atom in the phenol molecules. Also, the swelling of plant material by water increases extractability by allowing the solvent to penetrate easily into the solid matrix (Galanakis et al., 2013). A hydro-alcoholic mixture was proved to be the best solvent compared to methanol, ethanol, water and acetone for the extraction of phenolic compounds from roots of black cohosh due to their polar protic nature to donate hydrogen and intermediate polarity (Mukhopadhyay et al., 2006). In this backdrop, the present work was envisaged focusing on the extraction of bioactive compounds from *P. tetrastromatica* using suitable hydro-ethanolic solvents with an objective to evaluate their antioxidant and antimicrobial activity.

Materials and Methods

Seaweed (*P. tetrastromatica*) was collected from Punta Mandvi beach, Ratnagiri, Maharashtra, India, and washed thrice with fresh water to remove adhering sand and epiphytes followed by shade drying. It was then transported to the laboratory and dried again using mechanical drier at 50°C for 4 h. Further, it was ground to fine powder and sieved through 0.8 mm mesh.

Preparation of hydroethanolic extraction was done according to the method of Chew et al. (2008) with slight modifications. Two-gram seaweed powder was mixed well with 100 ml of solvent (solid to liquid ratio 1:50). The solution was kept in dark inside the shaking incubator maintained at 140 rpm for 2 h and then centrifuged (Eltek Centrifuge, Electrocrafter, India) at 3000 rpm for 10 min. The supernatant was collected and concentrated using vacuum rotary evaporator at 50°C. Each extract was weighed and the extractive yield was expressed as g 100 g⁻¹ dry seaweed powder. The extract was dissolved in 10 ml suitable solvent and stored at -20°C for evaluating antioxidant and antimicrobial activities.

Total phenolic content (TPC) of crude extract was determined by the Folin-Ciocalteu method, de-

scribed by Lin & Ching (2007). The absorbance was measured at 750 nm using a spectrophotometer. The standard curve was generated with Gallic acid standard, and the TPC was expressed in terms of mg Gallic acid equivalents g⁻¹ (mg GAE g⁻¹) extract.

DPPH radical scavenging activity of the extracts was measured according to the method of Thaipong et al. (2006) and the absorbance was measured at 515 nm. An equal volume of distilled water in place of sample was used for blank measurement. The standard curve was produced using Trolox as standard (25 to 1000 µM). Results were expressed as µmoles of Trolox Equivalent g⁻¹ extract (µM TE g⁻¹).

The ABTS radical scavenging assay was carried out by the method of Sumczynski et al. (2015) and absorbance was measured at 734 nm. Trolox was used as standard and the results were expressed as µM TE g⁻¹ extract.

The FRAP assay was done following the method of Thaipong et al. (2006). The absorbance of the reaction mixture was read at 593 nm. Results were expressed in µM TE g⁻¹ extract.

The total antioxidant activity of crude extracts was evaluated by phosphomolybdenum method of Thanigaivel et al. (2015) and absorbance was measured at 675 nm. Ascorbic acid was used as a standard (50 - 1000 ppm). Total antioxidant activity was expressed as mg ascorbic acid (AA) g⁻¹ extract.

The extracts were evaluated for their antimicrobial effects against seven common foodborne pathogenic and spoilage organisms including histamine formers, by disc-diffusion assay as described by Rajauria et al. (2011). Five Gram-negative (*Escherichia coli* O157: H7 (KAM 32), *Pseudomonas aeruginosa*, *Morganella morganii*, *Proteus vulgaris* and *Klebsiella pneumoniae* (laboratory repository isolates) and two Gram positive (*Bacillus subtilis*, MTCC 2757 and coagulase positive *Staphylococcus aureus*, ATCC 25923) bacteria were used. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 40 mg ml⁻¹. Young cultures were prepared from type cultures by incubating them in Muller Hinton broth for 3-4 h at 37°C and the optical density was adjusted to 0.5 McFarland standard. The bacteria were uniformly spread on sterile Muller Hinton agar plates using a sterile cotton swab. Whatman no.1 filter paper discs of 6 mm diameter were placed individually on each plate and were

impregnated with 15 μ l filter sterilized extract. The plates were incubated at 37°C for 18–24 h and the inhibition zones (in mm) including the disc dia. were measured. DMSO was used as negative control.

Bioactive compounds extracted from different extracts of *P. tetrastromatica* were separated on Hypersil gold column with 3 μ internal dia. The method was followed as described by Figueroa et al. (2018) with slight modification in operating conditions. Mobile phase was constituted by water and acetonitrile as solvent A and B respectively. The following multistep linear gradient elution programme was carried out to achieve efficient separation: 0.0 min (A: B, 95/5); 20.0 min (A: B, 0/100) 26.0 min (A: B, 95/5) and held until the end of run of 30 min. The compounds were identified based on their retention time in the column and the mass spectra database.

Data were analysed by One-way Analysis of Variance (ANOVA) using SPSS (Version 22, SPSS Inc., Chicago) and significant difference ($p \leq 0.05$) was determined by Duncan's Multiple Range Test.

Results and Discussion

The extraction yield measures the solvent's efficiency to extract specific components from seaweed powder. The results obtained for extraction yield and TPC of water, ethanol and their mixtures are depicted in Table 1. The yield percent varied among the extracts from $3.48 \pm 0.06\%$ to $25.26 \pm 0.1\%$. Water as an extraction solvent exhibited the highest extraction efficiency, while ethanol had the lowest efficiency. Tsakona et al. (2012) also reported lowest yield in absolute ethanol compared to other hydro-ethanolic mixtures while extracting phenolics from Mediterranean aromatic plants.

Estimation of TPC could be used as a rapid screening test of antioxidant activity of plant extracts as both are positively correlated with each other. The results obtained for TPC of all the extracts were significantly different from each other with 100% ethanolic extract having higher mean TPC value (68.69 ± 0.37 mg GAE g^{-1}), followed by hydro-ethanolic mixtures in the order of their increasing polarity. The aqueous extract had the lowest mean TPC value (10.37 ± 0.012 mg GAE g^{-1}). A similar observation was made by Fernando et al. (2016) who reported that the solvents with more aqueous content had low TPC owing to the presence of

greater number of simple polyphenols with low reduction potential. Although the yield of crude extract obtained in 100% ethanol was the lowest, the higher TPC value of this extract could be due to the presence of alkaloids in it which can readily reduce folins reagent to form blue complex. Folins reagent does not measure only phenols but will react with any reducing substance present in it, therefore, estimates the total reducing capacity of a sample, not just phenolics (Silva et al., 2013). Dang et al. (2018) reported a much higher TPC value (124.65 ± 0.78 mg GAE g^{-1}) for 70% ethanolic extract of *Padina* sp. collected from Bateau Bay, Australia. Such wide variation in TPC could be attributed not only to species variation but also to the geographical location and environmental conditions of their growth (Cho et al., 2010; Escrig et al., 2001).

The results obtained for different antioxidant assays for the extracts are presented in Fig. 1. In DPPH assay, the radical scavenging potential of the hydroethanolic extract is measured by the degree of discolouration of purple colour of DPPH radical, which is directly attributed to the hydrogen donating ability of the compounds present in the extract (Shimada et al., 1992). All the extracts showed antioxidant activity in varying degrees, significantly ($p \leq 0.05$) different from each other. Higher DPPH radical scavenging activity was obtained in absolute ethanol extract (114.66 ± 0.16 μ M TE g^{-1}) with highest TPC value and the lowest was observed in aqueous extract (9.38 ± 0.12 μ M TE g^{-1}). DPPH activity of extracts was hierarchical in the order of their decreasing polarity.

ABTS assay quantifies the ability of extract to scavenge cationic free radicals. The range obtained for cationic inhibition in the present study was too wide that it varied from 7.6 ± 0.23 μ M TE g^{-1} for aqueous extracts to 248.5 ± 1.22 μ M TE g^{-1} for ethanolic extract. Similar observation was reported by Thanigaivel et al. (2015) that ABTS scavenging activity of ethanolic extract of brown algae was much higher than that of aqueous extract. FRAP assay was carried out to measure the reducing capacity of antioxidant components to form ferrous (II) from ferric (III) by single electron transfer. The results from Fig. 1 showed that almost all the extracts had low FRAP values (11.02 ± 0.02 - 78.28 ± 0.0 μ M TE g^{-1}), while 100% ethanolic extract still indicated the highest. The total antioxidant activity of aqueous extract was measured as 6.13 ± 0.23 mg AA g^{-1} and that of ethanolic extract was 31.21 ± 0.67

mg AA g⁻¹ (p<0.05). However, there was no significant difference between the TAA of 20 and 40% extracts as well as 60 and 80%. The results obtained in the present study agree with the findings of Maheswari et al. (2017) who reported that the extracts of *P. tetrastomatica* collected from Mandapam coast, India exhibited low FRAP and TAA activity but a higher DPPH and ABTS activity at same concentration. Dang et al. (2018) also reported similar observations in ethanolic extracts of *Padina* sp. collected from Australia.

The minimum inhibitory zone measured against each test bacteria is presented in Table 2 and Fig. 2. Of the six extracts tested, 100% ethanolic extract showed the highest zone of inhibition (IZ) against *P. aeruginosa* (13.0±0.57 mm). Similarly, it also exhibited significant inhibition against *E. coli* with IZ of 10.3±0.33 mm. Whereas, Gram-positive bacteria like *S. aureus* and *B. subtilis* were more susceptible to the presence of 60% extracts with IZ of 10.0±0.0 mm and 9.33±0.33 mm respectively. These results are contrary to earlier reports which portrayed gram-positive bacteria, including *Bacillus* sp. and *Staphylococcus* sp., as more susceptible to seaweed phenolic extracts than gram negative bacteria due to their cell wall compositional variations (Shima et al., 2016; Rani et al., 2016; Dussault et al., 2016; Al-Enazi et al., 2018). However, gram negative histamine formers were found to be the most resistant among the test organisms. Sameeh et al., 2016 also observed the resistance of *Klebsiella oxytoca* against ethanolic extract of *Padina boryana*.

HR- LCMS analysis of the hydroethanolic extracts revealed the presence of various bioactive phenolics and alkaloids as presented in Table 3. Among phenolic compounds, phenolic acid groups like Everinic acid, 4- hydroxy phenyl propionic acid, 2-3 dichloro phenoxy acetic acid were present in water extract. The common major phenolic compound present in all the six extracts were 4-hydroxy phenyl propionic acid and Dienestrol. Etoposide is the phenolic compound which was present only in 40 and 60% extracts. The ethanolic and 80% extract contained less phenolics, but a greater number of different active alkaloid groups showed up in abundance, which could be the reason for the higher antioxidant activity observed in these extracts. Moreover, it reveals the increase in extraction efficiency towards alkaloid compounds as the ethanolic content in solvent mixture increases. Alkaloids are also reported to possess therapeutic

potential and was shown to be effective against several food-contaminating pathogens (Hussain et al., 2018; Hintz et al., 2015; Negi, 2012).

As water is highly polar than any other solvent, it can extract more polyphenols. Whereas, a decrease in polarity enables simultaneous extraction of phenolics and other bioactive compounds like terpenes and quinolines. This could be the actual reason for the significantly higher TPC and other antioxidant values observed in 100% ethanolic extract. Remarkably, a direct positive correlation could be observed between TPC and antioxidant activity of *P. tetrastomatica*. The present study suggests sulphated compounds along with polyphenols and alkaloids as principal constituents responsible for the antimicrobial properties of *P. tetrastomatica* extracts. An additional purification steps like sequential extraction with low polar solvent could be recommended to isolate the compounds of our interest.

Acknowledgements

The authors wish to thank the Director, ICAR-Central Institute of Fisheries Education, Mumbai and SAIF, IIT, Mumbai for providing all the facilities required for the research.

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