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Assessment of antioxidant activity in Victorian marine algal extracts using high performance thin-layer chromatography and multivariate analysis

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ABSTRACT

The aim of this study was to develop and validate a rapid and simple high performance thin layer chromatographic (HPTLC) method to screen for antioxidant activity in algal samples. 16 algal species were collected from local Victorian beaches. Fucoxanthin, one of the most abundant marine carotenoids was quantified directly from the HPTLC plates before derivatization, while derivatization either with 2,2-diphenyl-1-picrylhydrazyl (DPPH•) or ferric chloride ($FeCl_3$) was used to analyze antioxidants in marine algae, based on their ability to scavenge non biological stable free radical (DPPH•) or to chelate iron ions. Principal component analysis of obtained HPTLC fingerprints has classified algae species into 5 groups according to their chemical/antioxidant profiles. The investigated brown algae samples were found to be rich in non-and moderate-polar compounds and phenolic compounds with antioxidant activity. Most of the phenolic iron chelators also have shown free radical scavenging activity. Strong positive and significant correlations between total phenolic content and DPPH radical scavenging activity showed that, phenolic compounds, including flavonoids are the main contributors of antioxidant activity in these species. The results suggest that certain brown algae possess significantly higher antioxidant potential when compared to red or green algae and could be considered for future applications in medicine, dietary supplements, cosmetics or food industries. *Cystophora monilifera* extract was found to have the highest antioxidant concentration, followed by *Zonaria angustata, Cystophora pectinate*, *Codium fragile*, and *Cystophora pectinata*. Fucoxanthin was found mainly in the brown algae species. The proposed methods provide an edge in terms of screening for antioxidants and quantification of antioxidant constituents in complex mixtures. The current application also demonstrates flexibility and versatility of a standard HPTLC system in the drug discovery. Proposed methods could be used for the bioassay-guided isolation of unknown natural antioxidants and subsequent identification if combined with spectroscopic identification.

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1. Introduction

Due to their long and diverse evolutionary backgrounds, marine organisms offer vast genetic diversity and present a valuable source of bioactive compounds. Marine algae, although simple chlorophyll containing organisms, have extremely diverse morphological and reproductive features and produce a range of compounds with unique physiological and biochemical properties [1]. They are also able to grow in environmental extremes, where exposure to extreme light and high oxygen concentrations, leads to the increased formation of reactive oxygen species (ROS) [2]. Despite their exposure to these harmful ROS, healthy algae lack oxidative damage in their structural components (i.e. fatty acids), indicating the presence of protective antioxidant components in their cells (vitamins, pigments, and polyphenols) [3–5] that may also offer protection to the human body against ROS.

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide are natural byproducts of normal oxygen metabolism. Under normal circumstances, cells are able to defend themselves against ROS damage using enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase [6], and non-enzymatic antioxidants, like

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vitamins C and E and glutathione. However, ROS levels may increase beyond concentrations that can be compensated by the production of antioxidants, resulting in oxidative damage of cellular components, leading to the cell death and tissue injury. This is associated with the onset of a variety of chronic diseases in humans, including certain cancers [7] and inflammatory diseases [6]. Hence, consumption of antioxidants might help to neutralize these excess free radicals produced in the body. Current evidence strongly supports the contribution of phenolic compounds present in the diet, in the prevention of cardiovascular diseases (CVDs) and cancers, and also suggests they play a role in the prevention of neurodegenerative diseases and diabetes mellitus [8]. While the antioxidant benefits associated with the consumption of various terrestrial plants has long been accepted, the health benefits of consuming marine algae have not been widely recognized in Western counties.

Most polyphenols isolated from marine sources are from macro- and microalgae [9]. The structures of natural polyphenols vary from simple molecules, such as phenolic acids and other simple polyphenolic compounds, to the more complex phlorotannins, which consist of polymeric structures made up of units of phloroglucinol (1,3,5-trihydroxybenzene), which are found in *Phaeophyceae* (brown algae) [10,11]. Polyphenols exhibit a wide range of biological effects due to their antioxidant properties. Many of these phenolic compounds provide a chemical defense mechanism against predators. Moreover, the relatively high concentration of phenolic compounds in marine algae species contributes to their beneficial antioxidant properties. It is important to note that the antioxidant properties of many marine algae have been related to potential anti-aging, anti-inflammatory, anti-bacterial, anti-fungal, cytotoxic, anti-malarial, anti-proliferative, and anti-cancer effects [12,13].

Relatively little is known about the antioxidant properties of compounds derived from Victorian algae. Although there are publications on the antioxidant activity of numerous algal species commonly found in Australia, there are no reports of systematic testing for antioxidant activity. Spectrophotometric assays are commonly used for determination of total antioxidant activity and include; free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical, nitric oxide scavenging assay, superoxide free radical scavenging assay, and hydrogen peroxide radical scavenging. However, the disadvantage of these spectrophotometric methods is that they measure the total antioxidant capacity of the whole extract and not the antioxidant activity of the individual components present in the extract [14,15]. The time consuming isolation of individual compounds can be avoided if this type of assay is combined with chromatographic separation, either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC). However, the use of an on-line HPLC method has been reported not to be successful as slow reaction kinetics results in inaccurate measurements [16]. In contrast, the use TLC overcomes the disadvantages associated with the slow kinetics issue associated with the use of HPLC. When using TLC, many samples can be run simultaneously on the same plate, and therefore under the same experimental conditions, making analysis times short and reducing the cost. TLC combined with DPPH• assay in situ has been previously been used for the screening of antioxidants in marine bacteria [17], plant extracts [18], wine extracts [19,20], and herbal extracts [21]. In the TLC-DPPH• assay, a developed plate is sprayed or dipped in an alcohol DPPH• free radical solution. Resulting yellow spots against a purple background indicate the presence of an active antioxidant compound [18]. Compound identification can be achieved by either fingerprinting (analyzing the thin layer chromatogram) or by subsequent analysis using instrumental techniques (i.e. mass spectrometry).

The aim of this work was to develop a simple, fast method for screening algae extracts for antioxidant activity using the TLC method, combined with post-chromatographic derivatization with either $FeCl_3$ or DPPH• free radical, in order to quantify and compare both polyphenolic content and free radical scavenging activity. Principal component analysis (PCA) was applied to extract the features from the plate image (i.e. from sample fingerprints) and to provide full analytical information about chemical composition, similarity/dissimilarity between samples, and identified characteristic markers. PCA is a commonly used multivariate technique that is used to reduce multidimensional data set to 2D or 3D coordinates. PCA visualizes and classifies samples according to similarity, determines objects showing different properties from others (outliers), and defines important variables that can be used for data classification.

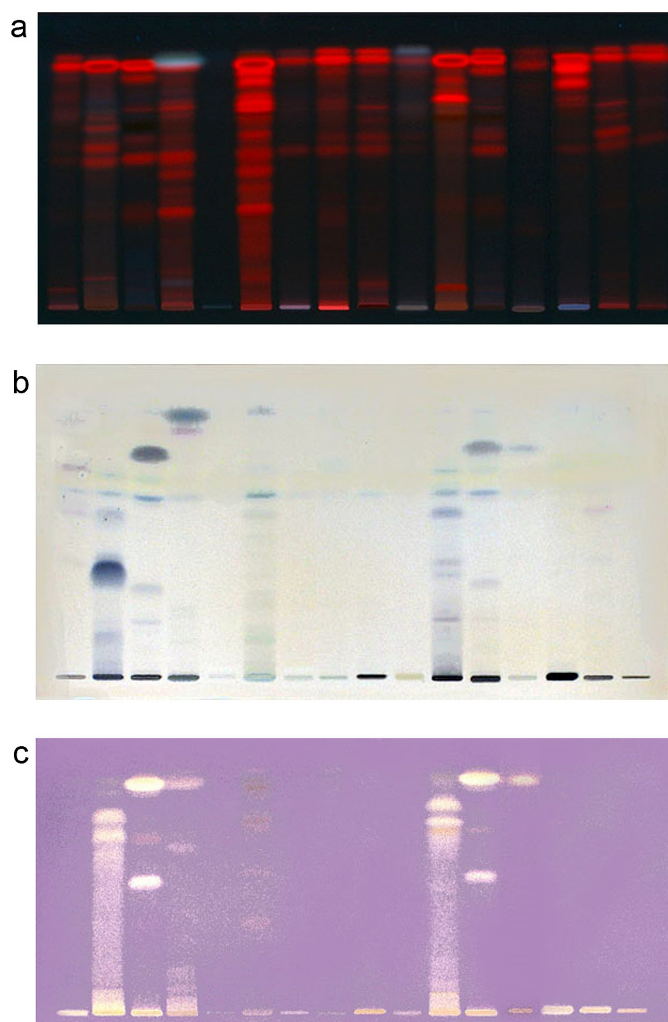

Fig. 1. HPTLC fingerprints of algal extracts (samples 1–16 from left to right) on normal phase HPTLC plates before derivatization and under 366 nm (a) and after post-chromatographic derivatization with (b) $FeCl_3$ and (c) DPPH•. Mobile phase, hexane: ethyl acetate: acetic acid (20:10:1). Fig. 1(a) photos taken under 366 nm, and Fig. 1(b) and (c) with white light above.

2. Material and methods

2.1. Chemicals used

2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) free radical, iron(III) chloride (97%), fucoxanthin (98%), and gallic acid (97%), were purchased from Sigma-Aldrich (Munich, Germany). All other solvents and chemicals used were of analytical grade. Acetic acid, acetone and methanol were purchased from Merck (Darmstadt,

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