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High-performance thin-layer chromatography HPTLC-direct bioautography as a method of choice for alpha-amylase and antioxidant activity evaluation in marine algae

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ABSTRACT

High-Performance Thin-layer chromatography (HPTLC) combined with DPPH• free radical method and α-amylase bioassay was used to compare antioxidant and antidiabetic activities in ethanol and ethyl acetate extracts from 10 marine macroalgae species (3 Chlorophyta, 4 Phaeophyta and 3 Rhodophyta) from Blue Lagoon beach (Malaysia). Samples were also evaluated for their phenolic and stigmasterol content. On average, higher antioxidant activity was observed in the ethyl acetate extracts (55.1 mg/100 g gallic acid equivalents (GAE) compared to 35.0 mg/100 g GAE) while, as expected, phenolic content was higher in ethanol extracts (330.5 mg/100 g GAE compared to 289.5 mg/100 g GAE). Amounts of fucoxanthin, stigmasterol and α-amylase inhibitory activities were higher in ethyl acetate extracts. Higher enzyme inhibition is therefore related to higher concentrations of triterpenes and phytosterols (Note: these compounds are more soluble in ethyl acetate). Ethyl acetate extracts from *Caulerpa racemosa* and *Padina minor*, had the highest α-amylase inhibitory activity, and also showed moderately high antioxidant activities, stigmasterol content and polyphenolic content. *Caulerpa racemose,* being green algae, does not contain fucoxanthin, while *Padina minor*, being brown algae, contains high amounts of fucoxanthin. Therefore, it is very unlikely that fucoxanthin contributes to α-amylase inhibitory activity as previously reported.

1. Introduction

Diabetes is a worldwide health challenge and economic burden to health care providers, due to our modern lifestyles and increased consumption of carbohydrate. One of the therapeutic targets in the management of diabetes is the inhibition of α-amylase, a digestive enzyme secreted from the pancreas and salivary gland [1]. Pancreatic α-amylase is involved in the breakdown of starch into disaccharides and oligosaccharides and release of glucose, which is then absorbed into the bloodstream. The inhibition of α-amylase reduces the breakdown of starch in the gastro-intestinal tract and subsequent glucose absorption. This may also lead to a reduction in postprandial hyperglycemia levels.

Plants present an important source of chemical compounds with potential α-amylase inhibitory activity. Due to their great diversity, there has been a considerable focus on marine algae as a source of bioactive compounds with therapeutic activity. The antidiabetic activity of bioactive compounds from marine algae has been previously associated with the antioxidant properties of compounds such as polyphenols and bromophenols [2,3]. Thus, the majority of studies have focussed on phenolic compounds with α-amylase inhibitory activity. Within this group, flavonoids have shown the highest activities which is thought to be related to the number of hydroxyl groups in the molecule [4]. Brown algae are well known for a large variety of bioactive substances, such as fucoxanthin, polyphenolic compounds, and phytosterols [5]. Fucoxanthin rich extracts from the brown algae *Sargassum hemiphyllum* have been shown to strongly inhibit α-amylase, α-glucosidase, sucrase, and maltase activities, and also to stimulate insulin secretion in vitro [6]. Fucoxanthin is a major carotenoid present in the chloroplasts of brown algae, followed by violoxanthin, and then β-carotene [7,8]. Fucoxanthin has attracted a lot of interest due to its reported anti-cancer [9], anti-obesity, anti-inflammatory effects [10,11], anti-diabetic, anti-angiogenic [12], antioxidant [13,14], and cancer chemo protective activity. It is a structurally unique molecule due to

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the presence of an unusual allenic double bonded carbon (C=C=C), a 5,6-monoepoxide, and 9 conjugated double bounds [10,15].

Phytosterols, are a group of compounds related to cholesterol that are present in all plants [16]. They are usually found in plant cell membranes, where they play important roles, similar to cholesterol in humans. Phytosterols are recognized as important components of healthy diets due to their ability to reduce cholesterol absorption and low-density lipoprotein (LDL) concentrations in the blood [17]. A diet rich in phytosterols is recommended by the EFSA (European Food Safety Authority) and the FDA (The US Food and Drug Administration) as part of a dietary strategy to reduce the risk of coronary heart disease [18,19]. The most frequently found phytosterols are β-sitosterol, campesterol, and stigmasterol [20]. Research on stigmasterol has shown it has antioxidant, hypoglycemic and thyroid inhibiting properties [21].

Due to the increase in the number of patients suffering from diabetes and the limited number of anti-diabetic drugs on the market, the search for new compounds, especially from marine sources, has attracted much interest recently. Uncontrolled high blood sugar (≥ 7.0 mmol/L of fasting plasma glucose or 11.1 mmol/L of plasma glucose) [22] can lead to a number of diabetes complications that result in high morbidity and mortality rates. Hyperglycaemia induces excess generation of highly reactive free radicals due to auto-oxidation of glucose, resulting in oxidative stress, which further aggravates the development and progression of diabetes and its complications [23]. However, clinical trials with classic antioxidants have not shown any benefit for diabetic patients [24]. Since oxidative stress plays a major role in the development of diabetes complications, an antioxidant therapy in combination with anti-diabetic drugs may be a good approach to prevent diabetic complications.

Since algae live in habitats that can vary significantly, they have developed diverse defence strategies to protect them from harsh environmental conditions, such as extensive light and oxygen, that leads to the formation of free radicals and other strong oxidizing agents. The absence of oxidative damage in their structural components and their resistance to oxidation during storage suggest the presence of powerful antioxidative defence systems [25,26].

Therefore, we wanted to evaluate and to characterise antidiabetic and antioxidant activities in marine algae extracts in terms of α-amylase inhibition and free radical scavenging activity. We also wanted to compare the activities of ethanol and ethyl acetate algal extracts (as phytosterols are more soluble in ethyl acetate) to establish if α-amylase inhibitory activity is related to fucoxanthin content, antioxidant potential, and/or phytosterol content. Combination of bioassays with chromatography (direct bioautography) enables rapid characterisation and identification of compounds in complex raw samples according to their activity profile. Plate chromatograms are directly immersed into an enzyme solution, incubated up to several hours, and then the activity profile of the enzyme-substrate reaction can be visualized, recorded and assessed.

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%), gallic acid (97%), stigmasterol (95%) were purchased from Sigma-Aldrich (Munich, Germany). HTLC separations were performed on 20 × 10 cm normal phase Silica gel 60 F254 HPTLC glass plates (Merck, Darmstadt, Germany).

Acetic acid, and methanol were purchased from Merck (Darmstadt, Germany), *n*-hexane from BDH (Poole, England), ethyl acetate from Sigma-Aldrich (Munich, Germany), anisaldehyde from ACROS organics (New Jersey, USA). All these chemicals were analytical reagent grade.

2.2. Sample collection and extraction

Eight fresh algae samples (samples 1–8) were collected in approximate 0.2–0.5 kg wet weight quantities from Blue Lagoon beach, Teluk Kemang, Port Dickson, Malaysia, and transported in sea water in cooled insulated containers to our laboratory (Table 1). Two commercially dried algae samples (sample 9 and 10) were purchased from the local shop in Kuala Terengganu (Table 1). Within twenty-four hours of collection, fresh algae samples were thoroughly rinsed three times with filtered seawater. Samples were then photographed, divided into 50–200 g portions, frozen at −80 °C and then lyophilised in a LAB CONCO freeze-drier (Kansas City, Missouri). Freeze dried samples were ground to a fine powder. Approximately 5 g of finely ground sample was extracted 5 times by vigorous shaking for 15 min with 50 mL of solvent (either ethanol or ethyl acetate), in sealed glass stoppered conical flasks and filtered. The resulting solvents were combined and concentrated to approximately 10 mL using a Büchi rotary evaporator Model R-200 (Labortechnik AG, Switzerland), transferred into 25 mL volumetric flasks and adjusted to volume with solvent. Commercial samples were ground and extracted following the same procedure as fresh samples. All extracts were stored at 4 °C to minimize degradation. Samples species were identified based on morphological characteristics and compared with descriptions of the samples that were collected from the same location and classified previously by Asmida et al. [27] and confirmed by comparison with the AlgaeBase database [28].

2.3. High-performance thin-Layer chromatography

Plates were pre-washed before use with a blank run of methanol and activated by drying in an oven at 100 °C for 30 min. Sample extracts (20 μL) were sprayed on the HPTLC-plates as 6 mm bands by using the Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland), 8 mm from the lower edge, with 14 mm distance from each side, and a minimum distance of 2 mm between each track. For a five-point calibration, 1.0–20.0 μL/band of the 1 mg/mL standard solutions were applied. TLC plates were developed in an Automated Multiple Development Chamber (AMD2, CAMAG, Muttenz, Switzerland) with a mobile phase of *n*-hexane, ethyl acetate, acetic acid (20:9:1) over a 80 mm developing distance.

2.3.1. Post chromatographic derivatization

A 0.4% DPPH• solution was prepared in methanol (Merck, Darmstadt, Germany), stored at 2–8 °C, and protected from light. Neutralized ferric chloride solution was prepared by adding dilute sodium hydroxide solution to freshly prepared 2% ferric chloride solution in methanol, drop by drop until ferric hydroxide starts to precipitate. The solution was then filtered to remove the precipitate and the clear filtrate was used for derivatization [29]. The anisaldehyde reagent solution was freshly prepared by mixing anisaldehyde with a refrigerated solution of glacial acetic acid/concentrated sulphuric acid in methanol (a ratio of 0.5:10:5:85). The colourless solution was stored in refrigerator (if color develops the reagent must be discarded).

Derivatization was achieved by dipping (immersing) a HPTLC plate into the derivatizing agent for 1 s and with an immersion speed of 5 cm/s using the Chromatogram Immersion Device (CAMAG, Muttenz, Switzerland). Plates derivatized with DPPH• solution, were stored in dark for 30 min and then photographed. Plates derivatized with anisaldehyde-sulfuric acid and with ferric chloride were heated at 110 °C for 10 min. Images of the plates were

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