Journal of Photochemistry and Photobiology B: Biology 143 (2015) 52–60

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Structural elucidation, *in vitro* antioxidant and photoprotective capacities of a purified polyphenolic-enriched fraction from a saltmarsh plant

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ARTICLE INFO

Article history:
Received 17 July 2014
Received in revised form 3 December 2014
Accepted 11 December 2014
Available online 27 December 2014

ABSTRACT

In temperate saltmarshes, halophytic plants have to daily protect their internal tissues against sunlight and UV rays. Consequently, they develop adaptive responses such as the synthesis of secondary metabolites, including polyphenols. The present study focused on the biological activities of fractions enriched in polyphenols from *Salicornia ramosissima*. Three different extracts were obtained by purification processes to concentrate polyphenols: a crude hydroalcoholic extract, and two purified fractions: an ethyl acetate fraction (EAF) and an aqueous fraction. Phenolic and flavonoid contents, antioxidant (DPPH radical-scavenging activity, reducing activity, β-carotene linoleic acid system and the ORAC method) and sunscreen properties (Sun Protection Factor and UVA-Protection Factor) were assessed by *in vitro* tests. The purification process was effective in increasing phenolic and flavonoid contents as well as antioxidant and sunscreen capacities of the EAF. The EAF appeared to be a broad spectrum UV absorber. The chemical structure of 10 EAF polyphenols was elucidated using 2D NMR and mass spectrometry spectra. Furthermore, a correlation was observed between phenolic composition and biological activity. These findings are encouraging for the future use of *S. ramosissima* as a potential source of antioxidant and photoprotectant molecules for industrial applications.

1. Introduction

The epidermis of both flora and fauna species have adaptive mechanisms that play a key role in photoprotection, especially the outer cell layer, because this represents the first barrier against the environment. Excessive and chronic, repeated exposure to solar radiation, particularly solar mid-wave ultraviolet radiation (UV-B; 290–320 nm), are the primary cause of immunosuppression, premature aging, melanomas, and benign and malignant tumors, of the skin in human [1]. Skin diseases occur when UV-B radiation overwhelms the cutaneous antioxidant capacity of the skin [2] causing a photodynamic elevation of free radicals and subsequent photo-oxidative stress and damage [3]. As the mortalities from human skin cancers continue to rise [3], it is essential to find preventive measures against photoaging and cancer development of the skin. The use of sunblocks from childhood and oral or topical use of antioxidants, represent possible ways to lower the risk of skin diseases [1]. In addition, as recently reflected by sales of nutraceuticals and in the overall therapeutic use of traditional medicines [4], the development of natural products represents a viable alternative method of preventive skin health care [1], including cosmetic applications [5].

Excessive absorption of photons and UV radiation causes similar destructive disorders in plants as in animals and humans, such as the formation of free radicals and DNA damage including cyclobutane pyrimidine dimers and photoproducts [1,6]. Compared with fauna, plant photoprotection uses specific mechanisms like phytohormones (polyamines) and polyphenol synthesis to mitigate such damage [6]. Polyphenols, which belong to a large family of plant products, are considered as secondary metabolites [7] although they play a crucial role in many interactions between plants and their environment [8]. The nuclear structure of phenolics is based

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http://dx.doi.org/10.1016/j.jphotobiol.2014.12.018
1011-1344/

on aromatic rings that bear one or more hydroxyl substituents [9], giving polyphenols the capacity to absorb the entire UV-B spectrum of wavelengths and part of the UV-C and UV-A spectra [1].

Phenolic polymer metabolism is thought to have played an important role in the evolution from aquatic to land plants in a terrestrial UV environment [10]. Saltmarsh habitats, at the transition between aquatic and terrestrial ecosystems, are dominated by non woody salt-tolerant vascular plants in the temperate coastal zone [11]. Saltmarsh plants are known to accumulate high levels of phenolic substances [12]. We therefore chose *Salicornia ramosissima* J. Woods, a true saltmarsh halophyte, distributed worldwide [13], for the present study. This succulent and edible glasswort species can be grown in Brittany by soilless cultivation [14,15]. Numerous biological activities beneficial to human health have been reported in extracts of glassworts, such as antioxidant, antihyperlipidemic, antidiabetic, antithrombus, antimicrobial, anti-inflammatory, antiproliferative and anticancer properties; they are also a source of vitamin C and diureticum [16,17]. Within the *Salicornia* genus, these medically-related biological activities have mainly been demonstrated in the species *S. herbaceae L.* and *S. europeae L.*

The aim of this work was to examine a novel natural photoprotective and antioxidant semi-purified fraction from *S. ramosissima*, a species rich in polyphenols. Total phenolic compounds were quantified and *in vitro* assays were used to measure the antioxidant and radical scavenging activities. The photoprotection sunscreen capacity was also tested by measuring the Sun Protection Factor (SPF) and Protection Factor-UV-A (PF-UV-A) *in vitro*. Then, the major phenolic compounds in the active fraction were identified by 2-D nuclear magnetic resonance (NMR) and liquid chromatography coupled with mass spectrometry (LC-MS) analysis.

2. Material and methods

2.1. Reagents

The reagents Tween 40, $FeCl_3$ and acid trichloroacetic were obtained from VWR. Folin–Ciocalteu phenol reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and catechin were purchased from Sigma–Aldrich. All other chemicals used were of analytical grade.

2.2. Plant material

S. ramosissima belongs to the family Amaranthaceae and subfamily Salicornioideae. Species identification had been made based on taxonomic criteria on the sampled glasswort population for several years and has since been confirmed by a phylogenetic study within the tribe of the Salicornieae of the Northern Atlantic coast [18]. This succulent halophyte has an articulated, highly branching dark green stem with a height between 20 and 40 cm. *S. ramosissima* has three flowering cymes per bract that form a characteristic triangle with a large central and two similar lateral flowers. It becomes purplish red in summer [19]. Samples were collected, at Bouin, France (47°1′42″N, 1°58′53″O) in an open saltmarsh of Bourgneuf Bay in September 2009. Only the aerial parts of the plants were used. After washing (in deionized water), plant material was frozen, then freeze-dried (CHRIST BETA 1-8 LD), ground and finally stored in darkness at room temperature. This conditioning treatment was chosen with the aim of limiting the degradation of phenolic compounds [20].

2.3. Extraction and liquid–liquid purification processes

Extraction was performed on 15 g plant dry matter using hydroethanolic solvent (v/v, 1:1) at 40 °C for three shaken macerations of 2 h and two of 1 h. Ethanol solvent was removed at 40 °C by vacuum evaporation to obtain a crude extract (CE). After dichloromethane washings, two successive precipitations with acetone and then ethanol and a final washing with ethyl acetate, two semi-purified extracts, an aqueous fraction (AF) and an ethyl acetate fraction (EAF) were obtained using a protocol adapted from Stiger-Pouvreau et al., with the aim of concentrating polyphenols in the final EAF [21].

2.4. Total phenolic and flavonoid contents

The total phenolic content was quantified using the Folin–Ciocalteu procedure adapted to microplates [22]. The wells were filled with 20 μL of extract, 130 μL distilled water and 10 μL Folin–Ciocalteu reagent followed by 40 μL Na_2CO_3 (200 g L^{-1}). The microplate was placed at 70 °C for 10 min and then put on ice to stop the chemical reaction. Absorbance was measured at 620 nm (Multiscan MS, Labsystems). Phenolic content was quantified based on a standard curve of gallic acid and results were expressed in mg gallic acid g^{-1} of dried extract (DW).

Flavonoid content was determined by a method adapted for microplates from Dewanto et al. [23]. Following this procedure, 25 μL of extract were mixed with 100 μL distilled water and 10 μL $NaNO_2$ (5%; w/v). Plates were incubated 5 min at room temperature. Then, 20 μL of $AlCl_3$ (5%; w/v) were added and plates incubated for another 5 min. After addition of 50 μL NaOH (1 M), the absorbance was read at 492 nm. Flavonoid content was determined based on a catechin standard curve and results were expressed as mg catechin g^{-1} DW.

2.5. Antioxidant activities

Coupling several antioxidant assays can provide an overview of the antioxidant capacity of active extracts and thus represent the complexity of antioxidant mechanisms involved in biological systems; this is especially the case for natural antioxidants, which are generally multifunctional [24,25]. In our case, four assays were performed and, for each test, butylated hydroxyanisole (BHA), trolox and α-tocopherol were used as positive controls.

2.5.1. β-Carotene bleaching test (BCBT)

Antioxidant capacity of extracts was quantified with the β-carotene bleaching test [26,27]. The BCBT method implies proton transfer; it simulates the antioxidant capacity of extracts faced with oxidation mechanisms similar to lipidic peroxidation [25,27]. At first, a mixture constituted of 2 mL β-carotene solution in chloroform (0.1 mg mL^{-1}), 20 mg linoleic acid and 200 mg Tween 40 was evaporated under vacuum. The reagent solution (emulsion) was obtained after addition of 50 mL oxygenated distilled water. For the test, 180 μL of reagent was added to 12 μL of extract or control. The absorbance was read at 450 nm immediately and after 2 h at 50 °C. Antioxidant activity was expressed as IC50 (g L^{-1}), the concentration at which 50% of β-carotene mixture bleaching was inhibited. IC50 was obtained from a linear regression analysis of the curve of inhibition percentage as a function of sample concentration, as recommended by Ksouri et al. [28].

2.5.2. DPPH radical scavenging assay

The radical scavenging activity was determined with the DPPH (2,2-diphenyl-1-picrylhydrasyl) assay [29,30]. This non-specific assay tests the capacity of samples to scavenge the synthetic radical, DPPH. It has the advantages of being independent of sample polarity [27] and highly reproducible [31]. For this method, 100 μL of sample were added to 100 μL of DPPH solution (36.9 mg L^{-1}) in a microplate. Absorbance was measured at 540 nm after 60 min of incubation in the darkness at room