Journal Of Applied Phycology
December 2016, Volume 28 Issue 6 Pages 3547-3559
http://dx.doi.org/10.1007/s10811-016-0853-0
http://archimer.ifremer.fr/doc/00366/47682/

Sunscreen, antioxidant, and bactericide capacities of phlorotannins from the brown macroalga *Halidrys siliquosa*

Le Lann Klervi [1, *], Surget Gwladys [1], Couteau Celine [2], Coiffard Laurence [2], Cerantola Stephane [3], Gaillard Fanny [4], Larnicol Maud [5], Zubia Mayalen [6], Guerard Fabienne [1], Poupart Nathalie [1], Stiger-Pouvreau Valerie [1]

[1] UBO, European Inst Marine Studies IUEM, LEMAR UMR UBO CNRS Ifremer IRD 6539, Technopole Brest Iroise, F-29280 Plouzane, France.
[2] Nantes Atlant Univ, Univ Nantes, Fac Pharm, LPiC,MMS,EA2160, 9 Rue Bias,BP 53508, F-44000 Nantes, France.
[3] UBO, RMN RPE MS, 6 Ave,Victor Le Gorgeu CS93837, F-29238 Brest 3, France.
[4] CNRS, Plateforme Spectrometrie Masse MetaboMER, FR2424, Stn Biol, Pl Georges Teissier,BP 74, F-29682 Roscoff, France.
[5] Venelle Carros, Labs Sci & Mer, CS 70002, F-29480 Le Relecq Kerhuon, France.
[6] Univ Polynesie Francaise, EIO UMR 244, LabEx CORAIL, BP 6570, Faaa 98702, Tahiti, Fr Polynesia.

* Corresponding author : Klervi Le Lann, email address : klervi.lelann@gmail.com

Abstract :

The present study focused on a brown macroalga (*Halidrys siliquosa*), with a particular emphasis on polyphenols and their associated biological activities. Two fractions were obtained by liquid/liquid purification from a crude hydroethanolic extract: (i) an ethyl acetate fraction and (ii) an aqueous fraction. Total phenolic contents and antioxidant activities of extract and both fractions were assessed by in vitro tests (Folin–Ciocalteu test, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power assay, superoxide anion scavenging assay, and β-carotene–linoleic acid system). For the most active fraction, i.e., the ethyl acetate fraction, the oxygen radical absorbance capacity (ORAC) value, antibacterial activities, and sunscreen potential (Sun Protection Factor and UV-A-Protection Factor) were tested in vitro. A high correlation found between antioxidant activities and total phenolic content was interpreted as the involvement of polyphenolic compounds in antioxidant mechanisms. Interestingly, the ethyl acetate fraction appeared to be a broad-spectrum UV absorber and showed a strong bactericidal activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. In this fraction, four phenolic compounds (trifuhalols and tetrafuhalols and, for the first time, diphlorethols and triphlorethols) were identified using 1D and 2D nuclear magnetic resonance (NMR) and MS analysis. These findings are promising for the use of *H. siliquosa*, abundant in Brittany, as a valuable source of photoprotectant molecules for sunscreen and cosmetic applications.

Keywords : Antioxidant, Bactericidal, Phenolic compounds, Phaeophyceae, Seaweed, ORAC value, Sunscreen potential

Introduction

One of the consequences of global change is an increase in the ambient level of ultraviolet (UV) radiation, particularly in southern areas (resulting from ozone depletion) (Thomas et al. 2012). This phenomenon, in addition to chronic sun exposure behavior, induces various skin-related disorders in humans, such as premature aging and skin cancer development (Lautenschlager et al. 2007; Thomas et al. 2012; Saewan and Jimtaisong 2015). UV radiation mostly consists of UV-A radiation, which penetrates deep into the dermis and epidermis and is known to be responsible for the production of reactive oxygen species (ROS), which can cause DNA damage. UV-A is also known to cause premature skin aging by damaging the underlying structures of the dermis (Ichihashi et al. 2003; Lautenschlager et al. 2007). UV-B radiation, which represents a lesser proportion of the total UV radiation than UV-A, is known to be the solar light component the most inductive of skin cancer, and can also cause DNA damage. It penetrates into the epidermis layer and causes some acute biological effects such as skin sunburn (Ichihashi et al. 2003; Lautenschlager et al. 2007). In order to lower the risk of skin diseases, topical sunscreen applications have been developed (Ichihashi et al. 2003; Lautenschlager et al. 2007; Sambandan and Ratner 2011). An optimal sunscreen combines UV-A and UV-B filters in order to provide broad spectrum protection (Lautenschlager et al. 2007; Sambandan and Ratner 2011). Present UV filters are, however, based on chemical compounds and cause many side effects, such as irritant or allergic reactions, dermatitis and in a few cases, severe anaphylactic reactions (reviewed in Lautenschlager et al. 2007). Thus, biotechnological research is now focusing on natural compounds, such as phytochemicals, that show promising bioactivities for sunscreen applications (Saewan and Jimtaisong 2015), such as absorption and scattering properties and conservative actions respectively in sunscreen and other cosmetic products and are suitable to replace some synthetic compounds. Among the organisms recognized as valuable sources of diverse bioactive chemicals, seaweeds remain an under-exploited resource, especially in Brittany (Bourgougnon and Stiger-Pouvreau 2011). Indeed, marine brown algae synthesize polyphenols, known as phlorotannins, that present various chemical structures (Singh and Bharate 2006) and have a large spectrum of biological activities (Li et al. 2011) of interest for skin protection. For example, phenolic compounds could have some antimicrobial (Eom et al. 2012), antioxidative (Liu et al. 2011), anti-cancer, radioprotective and anti-inflammatory properties (Kim et al. 2009; Kang et al. 2013). Moreover, in brown seaweeds, phlorotannins are of special interest regarding to the UV radiation protective effect (Pavia and Brock 2000; Swanson and Druehl 2002; Schoenwaelder 2002; Huovinen and Gómez 2015).

Previous works on development of innovative natural products for preventive skin health care have been done by our laboratory (Surget et al. 2015). Among brown macroalgae which grow along the coasts of Brittany (France) and can form dense forests in the subtidal zone, *Halidrys siliquosa* has been reported to have a high phenolic content (Stiger-Pouvreau et al. 2014) and potentially useful biological properties, such as strong antioxidant and interesting anti-tumor activities (Zubia et al. 2009). Moreover, this species belongs to the Sargassaceae family, which is known to present a natural diversity in bioactive compounds and is usually used as a model system for chemotaxonomic studies (Kornprobst 2010; Stiger-Pouvreau et al. 2014).

The goal of this study was to show the sunscreen potential of phlorotannins from this marine brown alga, in particular to identify active compounds (phycochemicals) with several biological activities. It focused on antioxidative activities associated with antibacterial and photoprotection capabilities of the phenolic-enriched fraction. Moreover, the total phenolic content of this active fraction was quantified with the Folin-Ciocalteu assay and qualified by NMR and MS analysis.

Materials and Methods

Biological Material

Halidrys siliquosa (Linnaeus) Lyngbye (Ochrophyta, Phaeophyceae, Fucales, Sargassaceae) was collected on January 30 2013, from the sheltered side of the Porsmeur site (N48° 28' 51"; W4° 46' 8") near Lanildut (France). Apical and median parts of thalli were collected, while the holdfasts were left on place to minimize collection impact on the seaweed population. Immediately after collection, epiphytes of *H. siliquosa* were removed and parts of thalli were thoroughly washed with distilled water. The cleaned algae were cut into small fragments and homogenized to remove effects of intra- and inter-individual variability. Samples were freeze-dried for 72 h and ground into powder for extraction.

Crude extraction and Liquid-Liquid purification processes

As described below and on Figure 1, two fractions (an ethyl acetate fraction or EAF and an aqueous fraction or AqF) were obtained from the purification of the hydroethanolic crude extract (CE).

Crude Extraction

To obtain the CE, 30 g of powder of thalli obtained from the collected sample were extracted with 300 mL of ethanol/water (50/50, v/v) mixture (EtOH 50) in the dark at 40 °C for 2 h under rotary agitation (200 rpm). Sample was centrifuged at 3000 *g* at 10 °C for 10 min and supernatant was collected. Next, the pellet was again extracted twice with 300 mL EtOH 50 in the dark at 40 °C for 1 h under rotary agitation (200 rpm) and centrifugation process was repeated. Finally, 900 mL of supernatant were obtained, pooled and evaporated at 40 °C under vacuum to reach 100 mL of crude extract (CE).

Liquid-Liquid Purification Process

Crude extract was semi-purified in order to concentrate the phenolic compounds using a process already described (Stiger-Pouvreau et al. 2014). Briefly, the process consisted of three steps. The first step was three dichloromethane washings. The second step consisted in rinsing the aqueous phase (containing phlorotannins) with acetone, and then with ethanol. Finally, the third step was three ethyl acetate rinses that permitted to obtain the aqueous (AqF) and the ethyl acetate (EAF) fractions. All further analyses were carried out on the crude extract and these two purified fractions.

Total Phenolic Content

Total phenolic content (TPC) was determined by spectrophotometry using an adapted Folin-Ciocalteu assay as described by Le Lann et al. (2008) and Zubia et al. (2009). The samples (100 µL) were mixed with 50 µL Folin-Ciocalteu reagent, 200 µL 20% sodium carbonate solution, and 650 µL distilled water. Then, the mixture was allowed to stand at 70 °C in the dark for 10 min. After a blue colour was produced, the

absorbance was read at 650 nm. The total phenolic compounds content (TPC) was expressed in mg per g of dry weight ($mg.g^{-1}$ DW) from a standard curve of phloroglucinol (1,3,5-trihydroxybenzene). This analysis was made in triplicate for each extract.

Antioxidant Assays

DPPH Radical Scavenging Activity

The DPPH radical-scavenging activity was determined by the method described by Zubia et al. (2009), modified from Fukumoto and Mazza (2000) and Turkmen et al. (2007). Briefly, 22 µL of CE and both fractions at various concentrations were mixed with 200 µL of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (25 $mg.L^{-1}$) prepared daily. The reaction was allowed to develop for 60 min in the dark at room temperature, before absorbance was read at 540 nm with a multi-well spectrophotometer. Water was used as a negative control, and ascorbic acid (Vitamin C), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2(3)-*t*-Butyl-4-hydroxyanisole (butylated hydroxyl-anisole or BHA) as positive controls. This assay was done in triplicate for each sample. The percentage of DPPH inhibition was calculated using the equation published in Surget et al. (2015). For each sample, a curve of extract concentration against % of DPPH inhibition was generated to estimate the concentration of extract needed to cause a 50% reduction of the initial DPPH concentration. IC_{50} was expressed in mg per mL. A high IC_{50} is considered as indicative of a weak radical scavenging activity and *vice versa.*

Reducing Power Assay

Total antioxidant capacity of the seaweed extract was determined using the ferric reducing-antioxidant power assay, as described by Zubia et al. (2009) and slightly modified by Surget et al. (2015). Briefly, 25 µL of sample at different concentrations were mixed with 25 µL sodium phosphate buffer (0.2M, pH 6.6) and 25 µL 1% potassium ferricyanide [$K_3Fe(CN)_6$]. After homogenization and incubation at 50 °C for 20 min, the mixture was cooled down in an ice bath prior to the addition of 25 µL 10% trichloroacetic acid, 100 µL deionised water, and 20 µL 0.1% $FeCl_3.6H_2O$. After homogenization, the reaction was allowed to develop for 10 min at room temperature and the absorbance was then read at 620 nm on a multi-well spectrophotometer. An increase of the absorbance at 650 nm of the reaction mixture indicated that the molecules had some reducing power. Results are expressed as EC_{50} in mg per mL. This value was obtained by interpolation of a regression linear curve. This assay was carried out in triplicate for each sample and for the positive controls (ascorbic acid, Trolox, and BHA, as described above).

Superoxide Anion-Scavenging Activity (NBT)

The superoxide anion scavenging assay was carried out according to Chua et al. (2008), as slightly modified by Tanniou et al. (2014). The reaction mixture consisted of 203 µL 5.10^{-3} M Tris–HCl buffer (pH 7.5), 57 µL 5mM hypoxanthine, 30 µL 0.33 mM NBT, and 13 µL of the extracts, positive controls (ascorbic acid, Trolox, and BHA) or negative controls (distilled water and ethanol). After incubation at 25 °C for 10 min, the reaction was started by adding 30 µL xanthine oxidase. The absorbance was measured every 3 min for 21 min at 560 nm. The inhibition ratio (%) was calculated from the equation published in Chua et al. (2008). Results were then expressed as IC_{50} in mg per mL (the concentration of substrate that causes a 50 % inhibition).

β-Carotene–Linoleic Acid System Test

The antioxidant activity of samples was measured by the β-carotene bleaching method in accordance with Kaur and Kapoor (2002) and Koleva et al. (2002) after slight modifications, as described in Le Lann et al. (2008) and Zubia et al. (2009). Briefly, 2 mL of a solution of β-carotene in chloroform (0.1mg.mL^{-1}) were added to round-bottom flasks containing 20 mg linoleic acid and 200 mg Tween 40. After evaporation with a rotavapor, oxygenated distilled water (50 mL) was added and the mixture was shaken to form a liposome solution. This mixture was added to 12 µL of extracts, positive controls (α-tocopherol, Trolox, and BHA) or negative controls (distilled water and ethanol). The absorbance of the solution at 450 nm was measured immediately (t = 0 min) and after 2 hours at 50 °C (t = 120 min). All samples were assayed in triplicate. Antioxidant activity was expressed through the antioxidant activity coefficient (AAC700 in mg per mL) described by Le Lann et al. (2008). Furthermore, the lower the AAC700 was, the stronger the antioxidant activity became.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC assay summarizes two results in a single value: the inhibition percentage and inhibition rapidity of peroxyl radicals by antioxidants, in competition with the substrate (Dudonné et al. 2009). The ORAC assay was performed by INVIVO LABS company (www.invivo-labs.com) on the most antioxidant fraction. The protocol was adapted from Cao et al. (1993). Results were expressed as µmol Trolox equivalent (Te) per mg.

Antibacterial Activity

Antibacterial assays were performed by IDEA LAB company (www.groupeideatests.com), Plouzané, France. The most antioxidant fraction was tested against bacteria at 5 mg.mL^{-1} in 1% EtOH. The three tested bacterial strains, referenced in the European Pharmacopoeia (2011), were: *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 8739). According to the benchmarks used, a product is bactericidal if a reduction of 4 or 5 log (AFNOR and European standards) of the bacterial concentration is obtained.

Protective Efficacy: Sun Protection Factor (SPF) and UV-A Protection Factor (PF-UV-A)

The photoprotective efficiency of *H. siliquosa* extracts was determined in UV-A and UV-B ranges (respectively 320-400 nm and 290-320 nm) using a previously described *in vitro* method (Couteau et al. 2007; El-Boury et al. 2007). SPF is used as a universal indicator to give information about the capacity of a product to reduce UV-induced solar erythema. An *in vitro* method makes it possible to highlight the UV filter role of organic or inorganic substances, which could later be incorporated into a sunscreen formulation for topical application.

Concisely, extracts were incorporated in a basic O/W (Oil in Water) emulsion to finally obtain an emulsion at 10% (w/w) of extracts. Fifty milligrams of prepared emulsion were spread using a cot-coated finger across the entire surface (25 cm^2) of a polymethylmethacrylate (PMMA) plate (Europlast, Aubervilliers). After spreading, only 15 mg of the emulsion remained on the plate. Transmission measurements between 200 and 400 nm were performed using a spectrophotometer equipped with an

integrating sphere (UV Transmittance Analyzer UV1000S, Labsphere, North Sutton, USA). Three plates were prepared for each extract to be tested and nine measurements were carried out on each plate. SPF and PF-UV-A were calculated using the following equations:

$$SPF = \frac{\sum_{290}^{400} E\lambda S\lambda d\lambda}{\sum_{290}^{400} E\lambda S\lambda T\lambda d\lambda} \qquad PF-UV\text{-}A = \frac{\sum_{320}^{400} E\lambda S\lambda d\lambda}{\sum_{320}^{400} E\lambda S\lambda T\lambda d\lambda}$$

where E_λ is erythemal spectral effectiveness at λ, S_λ is solar spectral irradiance at λ, and T_λ is spectral transmittance of the sample at λ (Diffey and Robson 1989).

Results were given as SPF and PF-UV-A. A higher SPF/PF-UV-A value indicates that a sample offers more protection against UV-B/UV-A by absorbance or reflection.

Structural Elucidation of Active Molecules by Nuclear Magnetic Resonance and MALDI-TOF Mass Spectrometry Analysis

The most antioxidant fraction was analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The 1D ^{1}H- and ^{13}C-NMR heteronuclear multiple bond correlation (HMBC) and heteronuclear single-quantum correlation spectroscopy (HSQC) experiments were carried out on a Bruker Avance 500. Samples were dissolved in 750 µL MeOD. Homonuclear and heteronuclear NMR spectra were recorded at 25 °C. Chemical shifts were measured in ppm using tetramethylsilane (TMS) as a chemical shift reference at 0 ppm.

Analysis by MALDI-TOF MS was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). The most active fraction (EAF) of *H. siliquosa* was first diluted to 1 $mg.mL^{-1}$ in water. Then 1 µL of this solution was diluted with matrix at 1/1, 1/10, 1/100 and 1/1000, and spotted onto the MALDI target. The matrix solution consisted of 2,5 dihydroxybenzoic acid (Sigma) 10 $mg.mL^{-1}$ in 50% ACN, 0.1% TFA. Spectra were acquired in positive ion reflector mode under 20 kV accelerating voltage, a delay time of 80 sec, and a mass range of 100–5000 Da. Each spectrum was the sum of 10000 single laser shots randomized over 10 positions within the same spot (1000 shots/position) at a laser frequency of 20 Hz. External calibration was performed using the Peptide mix 4 calibration mixture (Laser Bio Labs).

Statistical Analysis

The software R (v. 2.12.0) for Windows was used, with the RStudio (v. 0.95.263) integrated development environment. All laboratory analyses were performed in triplicate, and results were expressed as mean values ± standard deviation (SD). As data did not respect the requirements for ANOVA (homogeneity of variances, tested with the Bartlett's test at the 0.05 significance level), Kruskal-Wallis tests were performed, at a significance level of 95%, to reveal potential significant differences. In the case of significant differences between the data, non-parametric multiple comparisons (Behrens-Fisher test) were applied using the nmpc package. Moreover, to test the correlation between phenolic content and antioxidant activity, principal component analysis (PCA) was performed with the factomineR package and cluster analysis with the fpc and pvclust packages. Pvclust is designed to assess the uncertainty in

hierarchical cluster analysis. For each cluster in the hierarchical clustering, quantities called *p*-values are calculated via multiscale bootstrap resampling. The *p*-value of a cluster indicates how strongly the cluster is supported by the data. Clusters with *p*-values larger than 95% are strongly supported by data.

Results

Total Phenolic Content

The purification of the crude extract of *Halidrys siliquosa* was efficient in generating a phenolic-enriched ethyl acetate fraction, as shown in Table 1, which compares the total phenolic contents (TPC) of the crude extract and semi-purified fractions. Phenolic compounds were strongly concentrated in EAF. Indeed, almost 98% of this fraction (975.73 ± 19.26 mg.g^{-1} DW) consisted of phenolic compounds. The last 2% is probably constitued of traces of fatty acids and carbohydrates, as it is shown by the ^{1}H NMR analysis (Figure 2). Conversely, AqF showed lower TPC (164.19 ± 7.58 mg.g-1 DW) than CE (182.47 ± 3.33 mg.g-1 DW; Kruskal-Wallis test: $p < 0.05$; Behrens-Fisher test).

Antioxidant activities

Table 1 shows results of the antioxidant activities of the crude extract and both semi-purified fractions compared with positive controls, assessed by DPPH radical-scavenging activity, reducing power assay, superoxide anion-scavenging activity, and the β-carotene bleaching method.

The crude extract and both fractions showed high DPPH radical-scavenging activities. EAF exhibited the lowest IC_{50} (0.020 ± 3.54E10^{-4} mg.mL^{-1}), close to that obtained for the positive controls (*e.g.*, 0.010 ± 4.82E10^{-5} mg.mL^{-1}for BHA). The other antioxidant assays confirmed the results obtained by the DPPH test. Indeed, in the reducing power assay, EAF showed a low EC_{50} compared with those of the positive controls ($p < 0.05$, Behrens-Fisher test; 0.058 ± 0.001 mg.mL^{-1} and 0.074 ± 0.002 mg.mL^{-1} for EAF and BHA, respectively). In the superoxide anion-scavenging test, the IC_{50} displayed by EAF (0.664 ± 0.019 mg.mL^{-1}) was equal to the IC_{50} obtained for BHA (0.664 ± 0.022 mg.mL^{-1}). In the β-carotene bleaching method, EAF showed the lowest AAC700 (0.206 ± 0.001 mg.mL^{-1}, $p < 0.05$, Behrens-Fisher test). It should be noted, however, that this AAC700 value is significantly higher than those of the positive controls (*e.g.*, 0.015 ± 0.001 mg.mL^{-1} for BHA) (Table 1). Despite this last result, EAF showed strong antioxidant activities. Furthermore, whatever the antioxidant assay considered, AqF always showed the lowest activity by exhibiting a high IC_{50} (DPPH and superoxide anion-scavenging tests), a high EC_{50} (reducing power assay) and a strong AAC700 (β-carotene bleaching method). These results meant that AqF showed a weak antioxidant activity.

The ethyl acetate fraction of *H. siliquosa* showed an interesting ORAC value of 5.39 ± 1.08 µmolTE.mg^{-1} (Table 1), which is comparable to the ORAC value measured for ascorbic acid (9.35 ± 0.63) mentioned by Huang et al. (2010).

Correlations between Antioxidant Activities and Phenolic Content

It is worth noting that the ethyl acetate fraction, with marked antioxidant activities, had the highest phenolic content (975.73 ± 19.26 mg.mL^{-1} DW) whereas the aqueous fraction, which showed low antioxidant activities, had the lowest phenolic content (164.19 ± 7.58 mg.mL^{-1} DW). These observations

were confirmed by statistical analysis. Cluster analysis revealed a significant association between TPC and antioxidant activities (100%). Moreover, non-significant associations were found between the two scavenging tests (DPPH radical-scavenging and superoxide anion-scavenging assays, 70%) and between the reducing power test and β-carotene bleaching method (61%). The principal component analysis (PCA, Figure 3) confirmed that antioxidant activities were correlated with the total phenolic content of the samples. Moreover, PCA showed that TPC values were strongly negatively correlated with EC_{50} obtained with reducing power. This means that the higher the measured TPC of a sample, the lower its EC_{50} and the stronger its antioxidant activity. Other antioxidant assays were less correlated with TPC. PCA also confirmed that DPPH radical-scavenging and superoxide anion-scavenging assays were positively associated.

Bactericidal Activities

Results of the antibacterial assays are reported in Table 2. The negative control (1% EtOH) had no effect on bacterial growth, whatever the pathogen. In contrast, at a concentration of 5 $mg.mL^{-1}$, EAF showed a strong bactericidal activity. Indeed, this fraction showed a reduction higher than 5 log of the initial bacterial concentration with *Pseudomonas aeruginosa* and *Escherichia coli* (< 1 $UFC.mL^{-1}$). Concerning *Staphylococcus aureu*s, EAF showed a bactericidal activity with a reduction of 4.5 log of the initial bacterial concentration (75 $UFC.mL^{-1}$).

Photoprotective Sunscreen Activity

The absorption spectrum of the *H. siliquosa* EAF shows a band with a maximum at 376 nm. Moreover, the O/W emulsion manufactured with this phlorotannin-enriched fraction (*Halidrys* emulsion) had a Sun Protection Factor (SPF) of 3.55 ± 0.29 and a PF-UV-A value of 2.20 ± 0.13.

Structural elucidation of active molecules

Nuclear Magnetic Resonance Analysis

The ^1H NMR spectrum of the ethyl acetate fraction of *Halidrys siliquosa* showed a broader distribution of the ^1H signals between 5.8 and 6.3 ppm, characteristic of phlorotannins (Figure 2). Observations from the HMBC experiments (Figure 4 and Table 3) confirmed the presence of polyphenolic structures. Indeed, the HMBC spectrum showed characteristic carbon atom resonances at (1) 96.10 ppm, corresponding to quaternary methine groups; (2) between 124 and 132 ppm, corresponding to diaryl-ether bonds (ether-linked phloroglucinol units); (3) between 142 and 148 ppm for additional OH functions other than the 1,3,5 OH groups originally present in each phloroglucinol unit; and finally, (4) between 152 and 159 ppm, signals for phenolic carbons (Figure 4 and Table 3). We can, therefore, hypothesize that fuhalol type units, and also phlorethol-type units, are present. Moreover, it is worth noting the absence of signals between 100 and 105 ppm indicative of the absence of aryl-aryl carbons and thus the absence of fucol-type units in this purified phenolic sample.

MALDI-TOF Mass Spectrometry

The MALDI-TOF analysis of the ethyl acetate fraction of *Halidrys siliquosa* revealed a molecular weight ranging from 222 to 763 Da (Table 4). It could be resumed that the EAF contained small

polyphenolic compounds with no more than six phloroglucinol units (oligomers). The peak-to-peak mass increments observed at 273.06 Da, 413.29 Da, 523.36 Da, and 537.38 Da could correspond to the formation of M+ Na adducts of (**1**) (250 Da), (**3**) (390 Da), and (**4**) (514 Da) (Table 4 and Figure 5). Moreover, the peak-to-peak mass increment observed at 375.03 Da could correspond to the formation of M+ H adducts of (**2**) (374 Da). The peak-to-peak mass increments observed at 343.31 and 365.30 Da could correspond to the formation of M+ H and M+ Na adducts of a compound with a native mass of about 342 Da that seems not to be a phloroglucinol oligomer. Table 4 gives the experimental and calculated masses of the polyphenolic oligomers identified for the EAF sample (Figure 5), together with their putative chemical structures. Masses of phlorethols and fuhalols were calculated using the respective chemical formula of a typical phlorethol and fuhalol (respectively $C_{6n}H_{4n+2}O_{3n}$ and $C_{6n}H_{4n+2}O_{3n+i}$, where n is the number of phloroglucinol unitsand *i* was the number of additional hydroxyl groups).

Discussion

The purification process used in the present study to purify phenolic compounds from *Halidrys siliquosa* was highly efficient to isolating oligophlorotannins. This procedure was also described as efficient for other Sargassaceae species belonging to the genera *Bifurcaria, Cystoseira* and *Sargassum* (Stiger-Pouvreau et al. 2014), for Fucaceae species as *Pelvetia canaliculata* and *Ascophyllum nodosum* (Ar Gall et al. 2015) and also for halophytes (Surget et al. 2015). The preliminary structural analyses of the ethyl acetate fraction, combined with data from the literature (McInnes et al. 1984; Cérantola et al. 2006) allowed the identification of four phenolic compounds: di - and triphlorethols and tri- and tetrafuhalols. Until now, little information has existed on the chemical nature of phenolic compounds produced by *H. siliquosa*. Fuhalols had already been described in this species (Glombitza and Sattler 1973; Sattler et al. 1977) but, as these studies were done on non-native compounds (peracetylated compounds), the true nature of the phlorotannins present in *H. siliquosa* was not fully explored. Moreover, the present study is the first time which identified phlorethol-type compounds in this species. Phlorethols are known to be present in other Sargassaceae species, such as in *Sargassum muticum* and some *Cystoseira* species from Brittany (see Stiger-Pouvreau et al. 2014 for a review). Moreover, the co-occurrence of fuhalols and phlorethols was already known in other Sargassaceae species, like *Cystoseira tamariscifolia* (Glombitza et al. 1975) and *Sargassum spinuligerum* (Keusgen and Glombitza 1995), but not in *H. siliquosa*. Additionally, the phenolic compounds identified in *H. siliquosa* in the present study were oligomers of phloroglucinol. These results are in line with previous data on Sargassaceae species, which produces low-molecular-weight phenolic compounds (Le Lann et al. 2012a; Le Lann et al. 2012b; Harnita et al. 2013; Jégou et al. 2015; Montero et al. 2016) rather than high-molecular-weight phlorotannins as observed in the Fucaceae and Laminariales (Wang et al. 2012; Shibata et al. 2015; Heffernan et al. 2015).

In order to characterize the antioxidant capacity of extracts and fractions of *H. siliquosa*, four fast, reliable and classical biochemical methods were used. Three of them (DPPH, NBT, and Reducing Power tests) represent a single electron-transfer (SET) reaction, whereas the β-carotene bleaching method (BCBM) represents a hydrogen atom transfer reaction (HAT) (Huang et al. 2005). The antioxidant molecules can act as inhibitors of lipid oxidation through various different mechanisms in addition to free radical trapping, *e.g.*, prevention of chain initiation, binding of transition metal ion catalysts or peroxide decomposition (Frankel and Meyer 2000). Thus, simple *in vitro* tests are an approach to evaluate the antioxidant capacity of samples, and the combination of four of them made it possible to by-pass the

inability of a one-dimensional test of antioxidant capacity to accurately mirror the *in vivo* complexity of interactions between antioxidants in foods and biological systems (Frankel and Meyer 2000).

Among the tested samples, the ethyl acetate fraction exhibited the highest antioxidant activity independent of what assay was used. Moreover, its antioxidant activity was equivalent to those displayed by the commercial antioxidants used as positive controls in this study (ascorbic acid, α-tocopherol, BHA, and Trolox) for the SET assays (DPPH, NBT, and reducing power). These results were reinforced by the ORAC value obtained for the ethyl acetate fraction. Indeed, the ORAC value of the EAF (5.39 ± 1.08 µmol TE/mg) was close to the ORAC values obtained for ascorbic acid in the literature (Huang et al. 2010; Ishimoto et al. 2012; Fujii et al. 2013). For the β-carotene bleaching method, the activity of the EAF is higher than those of commercial antioxidants. This test measures the activity of lipophilic molecules (Koleva et al. 2002; Le Lann et al. 2008), so the active compounds would therefore tend to be polar, or faintly apolar compounds. Moreover, in the β-carotene bleaching method, the system is complex, and the emulsified lipid in use introduces additional variables liable to affect the oxidation process (Zubia et al. 2009).

To our knowledge, this study is the second to provide evidence for the existence of high antioxidant activity in this seaweed species, following a screening of antioxidant and antitumor activities of non-identified phlorotannins (Zubia et al. 2009). However, our paper is the first to identify active purified native phlorotannins from *H. siliquosa*. Moreover, the antioxidant activities exhibited by the extract and fractions of *H. siliquosa* were positively correlated with their phenolic contents. Additionally, Fujii et al. (2013) evaluated the antioxidative properties of phlorotannins isolated from the brown alga *Eisenia bicyclis* (Laminariales, Lessoniaceae). These authors found H-ORAC values of phloroglucinol (20.38 ± 1.11 µmol TE / mg) and various eckols (H-ORAC values between 11.6 ± 1.2 and 20.62 ± 1.46 µmol TE / mg). In the same way, in 2010, Parys et al. (2010) published ORAC values for phloroglucinol and fucophlorethols isolated from *Fucus vesiculosus*. Despite the unusual units used by these authors, their results showed that phloroglucinol was a little more active than fucophlorethols, meaning that oligomers of phloroglucinol could be more active than polymers for this test. Thus, our present study highlights, for the first time, the antioxidative potential of small phenolic compounds (oligofuhalols and oligophlorethols) from *H. siliquosa*. This enhances previous results on the high antioxidant potential of phenolic compounds from brown seaweeds. Further studies should now be carried out to isolate each phenolic compound and test them separately, to study potential synergy between phytochemicals. Furthermore, the marked correlation between the reducing power and the total phenolic content could provide information on the antioxidant mechanism of phenolic compounds from *H. siliquosa*. Indeed, the reducing power determined in this work, depends on the redox potentials of the compounds present in the sample. Therefore, it can be predicted that the phlorotannins present in the *H. siliquosa* EAF show low redox potential and thus a high antioxidant efficiency against free radicals (peroxyl or hydroxyl radicals) (Zhu et al. 2002).

The EAF of *H. siliquosa* also showed bactericidal activities against three bacterial strains: *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. The antimicrobial activities of phenolic compounds from seaweeds are very well known (see Eom et al. 2012 for a review), including phenolic compounds from *H. siliquosa* (Sattler et al. 1977). The antibacterial activities of phlorotannins were reviewed by Li et al. (2011), but this is the first time that bactericidal activities have been reported for a phenolic-rich fraction from *H. siliquosa*. More specifically, phlorotannins from *Ascophyllum nodosum* showed bactericidal activities against *E. coli* (Wang et al. 2009). The bactericidal activities of phenolic

compounds seemed to be related to the number of hydroxyl groups (Smith et al. 2003; Wang et al. 2009) and to the degree of polymerization of phloroglucinol within phlorotannins (Nagayama et al. 2002). Moreover, Wang et al. (2009) suggested that condensed phlorotannins with high hydroxylation may inhibit bacterial growth by altering the cell membrane (Wang et al. 2009). The fuhalol compounds identified in the EAF of *H. siliquosa* have high hydroxyl functions. The oxidation potential of hydroxyl groups, well known in phlorotannins (Ragan and Glombitza 1986), could explain their bactericidal activity, by alteration of bacterial cell membranes. Because this work is a preliminary study, there is now a need to test the dose effect to identify the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the EAF. Nevertheless, such fraction is interesting and could be used in cosmetic formula itself as natural conservators.

The SPF obtained for the *Halidrys* emulsion is similar to eight UV pure synthetic filters authorized in the European Union, like homosalate for example, tested in O/W emulsion with the same method (Couteau et al. 2007). While UV-B protection is imperative, UV-A protection is now recognized as being equally essential. Indeed, if UV-B is considered as "burning rays", then UV-A can be considered as "aging rays" (Lautenschlager et al. 2007; Saewan and Jimtaisong 2015). With a maximal absorbance (376 nm) in the UV-A range (400–320 nm) and a PF-UV-A about 2.20 ± 0.13, the EAF of *H. siliquosa* exhibited an absorbance and a sunscreen activity similar to the synthetic filter Avobenzone (358 nm and 2.76 ± 0.31 for maximal absorbance and PF-UV-A respectively) (Lohézic-Le Dévéhat et al. 2013). So, the EAF obtained from *H. siliquosa* is interesting because it appears to be a broad-spectrum UV absorber, useful to be included as natural filter in solar cream for example.

The photoprotective capacities of plant extracts have been demonstrated in previous studies. Indeed, using a similar *in vitro* UV method, three sunscreen emulsions with ethyl acetate plant extract (10 wt.%) were tested *in vitro* and authors obtained SPF and PF-UV-A values ranging from 6.00 ± 0.42 to 9.88 ± 1.66 and from 3.64 ± 0.07 to 6.96 ± 0.21, respectively (Jarzycka et al. 2013). Likewise, using the same protocol as the present study, one acetonic extract of the lichen *Lasallia pustulata* was found to have SPF and PF-UV-A maxima of 5.52 and 2.45, respectively (Lohézic-Le Dévéhat et al. 2013). Furthermore, usnic acid, extracted from the lichen *Xanthoparmelia farinosa,* was described as the best UV-B filter tested, with a protection factor similar to a commercial product called LSF 5 (4.1; Rancan et al. 2002). Among marine macrophytes, the EAF from a *Salicornia ramosissima* extract is an interesting candidate to provide an effective protective action over the whole UV-A–UV-B range, with large SPF and PF-UV-A values (Surget et al. 2015). Moreover, the photoprotection efficiency of seaweed extracts has been demonstrated for several species, as recently reviewed by Saewan and Jimtaisong (2015). For example, the photoprotective potential of extracts obtained from 11 commercial brown algae and 10 commercial red algae was evaluated against UV-B radiation (Guinea et al. 2012). In the same way, phlorotannins isolated and purified from *Ecklonia cava* were demonstrated as effective at protecting against UV-B radiation (Cha et al. 2012). Both of these studies used an *in-vivo* test with a zebrafish (*Danio rerio)* embryo assay. In a more generalistic point of view, the commun feature of UV-absorbing secondary metabolites is the presence of aromatic or conjugated bond structures as it is found in phlorotannins. Such molecules presents a π-electron system, which is one of the most effective UV radiation absorbers (Cockell and Knowland 1999).

One other point that would be taken into consideration is the spatio-temporal variability of phenolic contents. Indeed, the phlorotannin pool strongly depends on sites,seasons and on the phenology of algae (*i.e.:* Parys et al. 2009; Le Lann et al. 2012a; Celis-Plá et al. 2016). So, knowledge of the chemical ecology

of potentially exploitable species is essential to identify the best sites and seasons for the highest bioactivities.

Conclusions

This study emphasized, for the first time, the sunscreen potential and strong antioxidant and antibacterial capacities of a mix of four small phlorotannins, *i.e.*, di and tri-phlorethols and tri- and tetra-fuhalols, from the brown macroalga *Halidrys siliquosa*. The antioxidant and sunscreen activities were found to be equivalent to several commercial antioxidant molecules and to some synthetic UV filters. Moreover, the correlation found between antioxidant activities and total phenolic content supports the involvement of phenolic compounds in the antioxidant mechanisms. Furthermore, the EAF showed bactericidal activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, which represents interesting properties for proposing these compounds as natural preservatives in cosmetics for example. These findings therefore highlight the potential of this brown seaweed as a natural source of phytochemicals with several biological activities of interest to both the cosmetics and pharmaceutical industries. Nevertheless, it would be worth carrying out additional experiments in order to evaluate the photostability of compounds over time, and to check for the absence of cytotoxicity so they can be used in formulations. Moreover, because our results suggest that bioactivities of the EAF are derived from the synergy between the active phlorotannins, other further studies need to be carried out to isolate and to separately test the bioactive compounds present in the active fraction in order to gain a better understanding of their mechanisms of action**.**

Acknowledgments: This work was co-financed with the support of the European Union ERDF - Atlantic Area Programme, MARMED project no. 2011-1/164 and the project RIV-ALG (n° 13006760), financed by CBB Development, and the region Bretagne (France). Authors from LEMAR would like to thank the Labex Mer initiative, a State Grant from the French Agence Nationale de la Recherche (ANR) in the "Investissements d'avenir" Program (reference ANR-10-LABX-19-01, Labex Mer). The first author thanks Alexandre Canton for his assistance during the extraction and purification process, and Helen McCombie for reviewing English.

Author Contributions: Klervi Le Lann, Valérie Stiger-Pouvreau and Laurence Coiffard conceived and designed the experiments; Klervi Le Lann, Gwladys Surget, Céline Couteau, Stéphane Cérantola and Fanny Gaillard performed the experiments; Klervi Le Lann analyzed the data; Mayalen Zubia, Fabienne Guérard, Nathalie Poupart and Valérie Stiger-Pouvreau contributed materials and analysis tools; Klervi Le Lann, Valérie Stiger-Pouvreau, Fabienne Guérard, Gwladys Surget, Stéphane Cérantola, Mayalen Zubia and Laurence Coiffard wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. This article does not contain any studies with live animals performed by any of the authors. All authors contributed significantly to this work and are in agreement with the content of the manuscript.

References

Ar Gall E, Lelchat F, Hupel M, Jegou C, Stiger-Pouvreau V (2015) Chapter 7. Extraction and purification of phenols (phlorotannins) from brown algae. In Natural Products from Marine Algae: Methods and Protocols, Eds. D.B. Stengel et S. Connan. Springer, © Springer Science+Business Media New York 2015. Vol. 1308: 131-143

Bourgougnon N, Stiger-Pouvreau V (2011) Chemodiversity and bioactivity within red and brown macroalgae along the French coasts, Metropole and Overseas Departements and Territories. In: Kim S-K (ed) Handbook of Marine Macroalgae. John Wiley & Sons, Ltd, pp 58–105

Cao G, Alessio HM, Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 14:303–311.

Celis-Plá PSM, Bouzon ZL, Hall-Spencer JM, et al (2016) Seasonal biochemical and photophysiological responses in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta). Mar Environ Res 115:89–97.

Cérantola S, Breton F, Gall EA, Deslandes E (2006) Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. Bot Mar 49:347–351.

Cha S-H, Ko C-I, Kim D, Jeon Y-J (2012) Protective effects of phlorotannins against ultraviolet B radiation in zebrafish (*Danio rerio*). Vet Dermatol 23:51–e12.

Chua M-T, Tung Y-T, Chang S-T (2008) Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophloeum*. Bioresour Technol 99:1918–1925.

Cockell CS, Knowland J (1999) Ultraviolet radiation screening compounds. Biol Rev Camb Philos Soc 74:311–345.

Couteau C, Pommier M, Paparis E, Coiffard LJM (2007) Study of the efficacy of 18 sun filters authorized in European Union tested *in vitro*. Pharm - Pharmazie 62 (6):449-452

Diffey BL, Robson J (1989) A new substrate to measure sunscreen protection factors throughout the ultraviolet spectrum. J Soc Cosmet Chem 40:127–133.

Dudonné S, Vitrac X, Coutière P, Woillez M, Mérillon JM (2009) Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J Agric Food Chem 57:1768–1774.

El-Boury S, Couteau C, Boulande L, Paparis E, Coiffard L J M (2007) Effect of the combination of organic and inorganic filters on the Sun Protection Factor (SPF) determined by in vitro method. Int J Pharm 340:1–5.

513 Eom S-H, Kim Y-M, Kim S-K (2012) Antimicrobial effect of phlorotannins from marine brown algae.
514 Food Chem Toxicol 50:3251–3255.

515 European Pharmacopoeia 7.0 - 2011. https://www.edqm.eu. Accessed 27 Oct 2015

516 Frankel EN, Meyer AS (2000) The problems of using one-dimensional methods to evaluate
517 multifunctional food and biological antioxidants. J Sci Food Agric 80:1925–1941.

518 Fujii Y, Tanaka R, Miyake H, et al (2013) Evaluation for antioxidative properties of phlorotannins
519 isolated from the brown alga *Eisenia bicyclis*, by the H-ORAC method. Food Nutr Sci 04:78–82. doi:
520 10.4236/fns.2013.48A010

521 Fukumoto LR, Mazza G (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. J
522 Agric Food Chem 48:3597–3604.

523 Glombitza K-W, Rosener H-U, Müller D (1975) Bifuhalol und diphlorethol aus *Cystoseira*
524 *tamariscifolia*. Phytochemistry 14:1115–1116.

525 Glombitza K-W, Sattler E (1973) Trifuhalol, ein neuer triphenyldiäther aus *Halidrys siliquosa* (1).
526 Tetrahedron Lett 14:4277–4280.

527 Guinea M, Franco V, Araujo-Bazán L, Rodríguez-Martín I, González S (2012) In vivo
528 UVB-photoprotective activity of extracts from commercial marine macroalgae. Food Chem Toxicol
529 50:1109–1117.

530 Harnita ANI, Santosa IE, Martono S, Sudarsono S, Widyarini S, Harren FJ (2013) Inhibition of lipid
531 peroxidation induced by ultraviolet radiation by crude phlorotannis isolated from brown algae
532 *Sargassum hystrix* v. *buxifolium* C. Agardh. Indones J Chem 13:14–20.

533 Heffernan N, Brunton NP, FitzGerald RJ, Smyth TJ (2015) Profiling of the Molecular Weight and
534 Structural Isomer Abundance of Macroalgae-Derived Phlorotannins. Mar Drugs 13:509–528.

535 Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem
536 53:1841–1856.

537 Huang W-Y, Majumder K, Wu J (2010) Oxygen radical absorbance capacity of peptides from egg white
538 protein ovotransferrin and their interaction with phytochemicals. Food Chem 123:635–641.

539 Huovinen P, Gómez I (2015) UV sensitivity of vegetative and reproductive tissues of two antarctic brown
540 algae is related to differential allocation of phenolic substances. Photochem Photobiol
541 91(6):1382-1388.

542 Ichihashi M, Ueda M, Budiyanto A, Bito T, Oka M, Fukunaga M, Horikawa T (2003) UV-induced skin
543 damage. Toxicology 189:21–39.

Ishimoto H, Tai A, Yoshimura M, Amakura, Y, Yoshida T, Hatano T, Ito H (2012) Antioxidative properties of functional polyphenols and their metabolites assessed by an ORAC assay. Biosci Biotechnol Biochem 76:395–399.

Jarzycka A, Lewińska A, Gancarz R, Wilk KA (2013) Assessment of extracts of *Helichrysum arenarium, Crataegus monogyna, Sambucus nigra* in photoprotective UVA and UVB; photostability in cosmetic emulsions. J Photochem Photobiol B 128:50–57.

Jégou C, Kervarec N, Cérantola S, Bihannic I, Stiger-Pouvreau V (2015) NMR use to quantify phlorotannins. The case of *Cystoseira tamariscifolia*, a phloroglucinol-producing brown macroalga in Brittany (France). Talanta 135:1-6.

Kang Y-M, Eom S-H, Kim Y-M (2013) Protective effect of phlorotannins from *Eisenia bicyclis* against lipopolysaccharide-stimulated inflammation in HepG2 cells. Environ Toxicol Pharmacol 35:395–401.

Kaur C, Kapoor HC (2002) Anti-oxidant activity and total phenolic content of some Asian vegetables. Int J Food Sci Technol 37:153–161.

Keusgen M, Glombitza K-W (1995) Phlorethols, fuhalols and their derivatives from the brown alga *Sargassum spinuligerum*. Phytochemistry 38:975–985.

Kim A-R, Shin T-S, Lee M-S, et al (2009) Isolation and identification of phlorotannins from *Ecklonia stolonifera* with antioxidant and anti-inflammatory properties. J Agric Food Chem 57:3483–3489.

Koleva II, Van Beek TA, Linssen JPH, Groot AD, Evstatieva LN (2002) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 13:8–17.

Kornprobst J-M (2010) Encyclopedia of Marine Natural Products. John Wiley & Sons

Lautenschlager S, Wulf HC, Pittelkow MR (2007) Photoprotection. The Lancet 370:528–537.

Le Lann K, Connan S, Stiger-Pouvreau V (2012a) Phenology, TPC and size-fractioning phenolics variability in temperate Sargassaceae (Phaeophyceae, Fucales) from Western Brittany: native vs. introduced species. Mar Environ Res 80:1–11.

Le Lann K, Ferret C, VanMee E, Spagnol C, Lhuillery M, Payri C, Stiger-Pouvreau V (2012b) Total phenolic, size-fractionated phenolics and fucoxanthin content of tropical Sargassaceae (Fucales, Phaeophyceae) from the South Pacific Ocean: Spatial and specific variability. Phycol Res 60:37–50.

Le Lann K, Jégou C, Stiger-Pouvreau V (2008) Effect of different conditioning treatments on total phenolic content and antioxidant activities in two Sargassacean species: Comparison of the frondose

574 *Sargassum muticum* (Yendo) Fensholt and the cylindrical *Bifurcaria bifurcata* R. Ross. Phycol Res
575 56:238–245.

576 Liu H, Zhao L, Guo S, Xia Y, Zhou P (2011) Modification of fish skin collagen film and absorption
577 property of tannic acid. J Food Sci Technol.

578 Li Y-X, Wijesekara I, Li Y, Kim S-K (2011) Phlorotannins as bioactive agents from brown algae. Process
579 Biochem 46:2219–2224.

580 Lohézic-Le Dévéhat F, Legouin B, Couteau C, Boustie J, Coiffard L (2013) Lichenic extracts and
581 metabolites as UV filters. J Photochem Photobiol B 120:17–28.

582 McInnes AG, Ragan MA, Smith DG, Walter JA (1984) High-molecular-weight phloroglucinol-based
583 tannins from brown algae: Structural variants. Hydrobiologia 116-117:597–602.

584 Montero L, Sánchez-Camargo AP, García-Cañas V, et al (2016) Anti-proliferative activity and chemical
585 characterization by comprehensive two-dimensional liquid chromatography coupled to mass
586 spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on
587 North-Atlantic coasts. J Chromatogr A 1428:115–125.

588 Nagayama K, Iwamura Y, Shibata T, et al (2002) Bactericidal activity of phlorotannins from the brown
589 alga *Ecklonia kurome*. J Antimicrob Chemother 50:889–893.

590 Parys S, Kehraus S, Krick A, Glombitza KW, Carmeli S, Klimo K, Gerhäuser C, König GM (2010) In
591 vitro chemopreventive potential of fucophlorethols from the brown alga *Fucus vesiculosus* L. by
592 anti-oxidant activity and inhibition of selected cytochrome P450 enzymes. Phytochemistry
593 71:221–229.

594 Parys S, Kehraus S, Pete R, Küpper FC, Glombitza KW, König GM (2009) Seasonal variation of
595 polyphenolics in *Ascophyllum nodosum* (Phaeophyceae). Eur J Phycol 44:331–338.

596 Pavia H, Brock E (2000) Extrinsic factors influencing phlorotannin production in the brown alga
597 *Ascophyllum nodosum*. Mar Ecol Process Ser 193:285–294.

598 Ragan MA, Glombitza K-W (1986) Pholorotannins, brown algal polyphenols. Prog Phycol Res
599 4:129–241.

600 Rancan F, Rosan S, Boehm K, Fernández E, Hidalgo ME, Quihot W, Rubio C, Boehm F, Piazena H,
601 Oltmanns U (2002) Protection against UVB irradiation by natural filters extracted from lichens. J
602 Photochem Photobiol B 68:133–139.

603 Saewan N, Jimtaisong A (2015) Natural products as photoprotection. J Cosmet Dermatol 14:47–63.

Sambandan DR, Ratner D (2011) Sunscreens: An overview and update. J Am Acad Dermatol 64:748–758.

Sattler E, Glombitza K-W, Wehrli FW, Eckhardt G (1977) Antibiotica aus algen—XVI: Polyhydroxyphenyläther aus der Phaeophycee *Halidrys siliquosa*. Tetrahedron 33:1239–1244.

Schoenwaelder MEA (2002) The occurrence and cellular significance of physodes in brown algae. Phycologia 41:125–139.

Shibata T, Nagayama K, Sugiura S, Makino S, Ueda M, Tamaru Y (2015) Analysis on Composition and Antioxidative Properties of Phlorotannins Isolated from Japanese *Eisenia* and *Ecklonia* Species. Am J Plant Sci 06:2510–2521.

Singh IP, Bharate SB (2006) Phloroglucinol compounds of natural origin. Nat Prod Rep 23:558–591.

Smith AH, Imlay JA, Mackie RI (2003) Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory ffects of condensed Tannins. Appl Environ Microbiol 69:3406–3411.

Stiger-Pouvreau V, Jégou C, Cérantola S, Guérard F, Le Lann K (2014) Phlorotannins in Sargassaceae Species from Brittany (France). In: Advances in Botanical Research. Elsevier, pp 379–411

Surget G, Stiger-Pouvreau V, Le Lann K, Kervarec N, Couteau C, Coiffard LJM, Gaillard F, Cahier K, Guérard F, Poupart N (2015) Structural elucidation, in vitro antioxidant and photoprotective capacities of a purified polyphenolic-enriched fraction from a saltmarsh plant. J Photochem Photobiol B 143:52–60.

Swanson AK, Druehl LD (2002) Induction, exudation and the UV protective role of kelp phlorotannins. Aquat Bot 73:241–253.

Tanniou A, Vandanjon L, Incera M, Serrano Leon E, Husa V, Le Grand J, Nicolas JL, Poupart N, Kervarec N, Engelen A, Walsh R, Guerard F, Bourgougnon N, Stiger-Pouvreau V (2014) Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga *Sargassum muticum* sampled along its European range from Norway to Portugal. J Appl Phycol 26:1215–1230.

Thomas P, Swaminathan A, Lucas RM (2012) Climate change and health with an emphasis on interactions with ultraviolet radiation: a review. Glob Change Biol 18:2392–2405.

Turkmen N, Velioglu YS, Sari F, Polat G (2007) Effect od extraction conditions on measured total polyphenol contents and antioxidant and antibacterial activities of Black Tea. Molecules 12:484–496.

633 Wang T, Jónsdóttir R, Liu H, Gu L, Kristinsson HG, Raghavan S, .la fsdóttir G (2012) Antioxidant
634 capacities of phlorotannins extracted from the brown algae *Fucus vesiculosus*. J Agric Food Chem
635 60:5874–5883.

636 Wang T, Jónsdóttir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal
637 chelation of extracts from Icelandic seaweeds. Food Chem 116:240–248.

638 Zhu QY, Hackman RM, Ensunsa JL, Holt RR, Keen CL (2002) Antioxidative activities of Oolong Tea. J
639 Agric Food Chem 50:6929–6934.

640 Zubia M, Fabre MS, Kerjean V, Le Lann K, Stiger-Pouvreau V, Fauchon M, Deslandes E (2009)
641 Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chem
642 116:693–701.

643

Figure 1. Procedure of liquid:liquid purification carried out on the hydroethanolic crude extract of *Halydris siliquosa*.

Figure 2. ^{1}H NMR spectrum of the ethyl acetate fraction of the brown macroalga *Halidrys siliquosa* (in MeOD), focusing on the aromatic region.

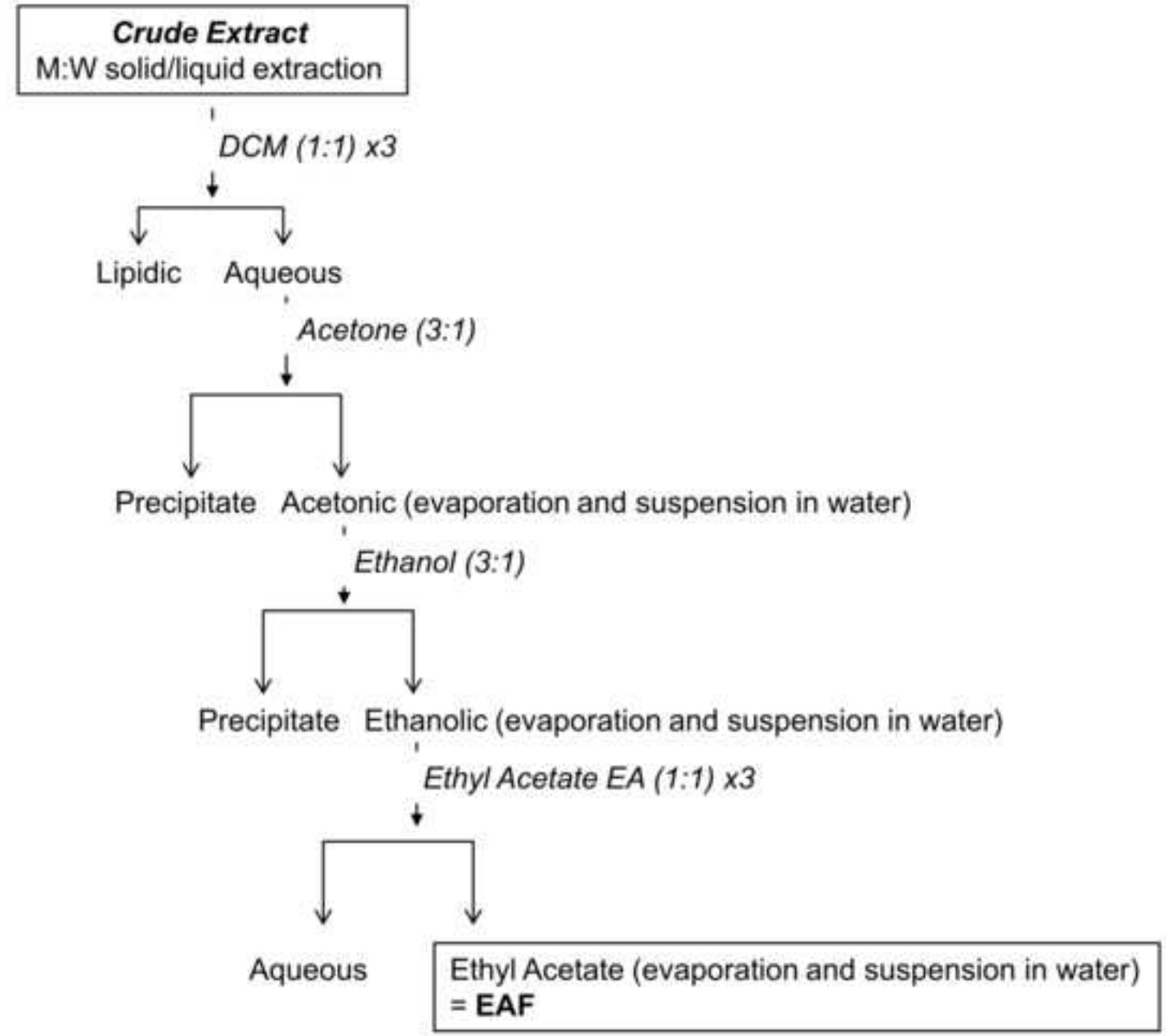
Figure 3. Principal component analysis (PCA), applied to the active variables of radical-scavenging activity (DPPH), reducing power assay (RP), superoxide anion-scavenging test (NBT) and the β-carotene bleaching method (BCBM).

Figure 4. HMBC contour plots of the ethyl acetate fraction of *Halidrys siliquosa* (in MeOD)

Figure 5. Chemical structures of phlorotannins identified in the ethyl acetate fraction of the brown alga *Halidrys siliquosa.*

Figure 1

Click here to download line figure Fig1.tiff



Adapted from Stiger-Pouvreau et al. 2014

Figure 2

Click here to download line figure Fig2.tiff

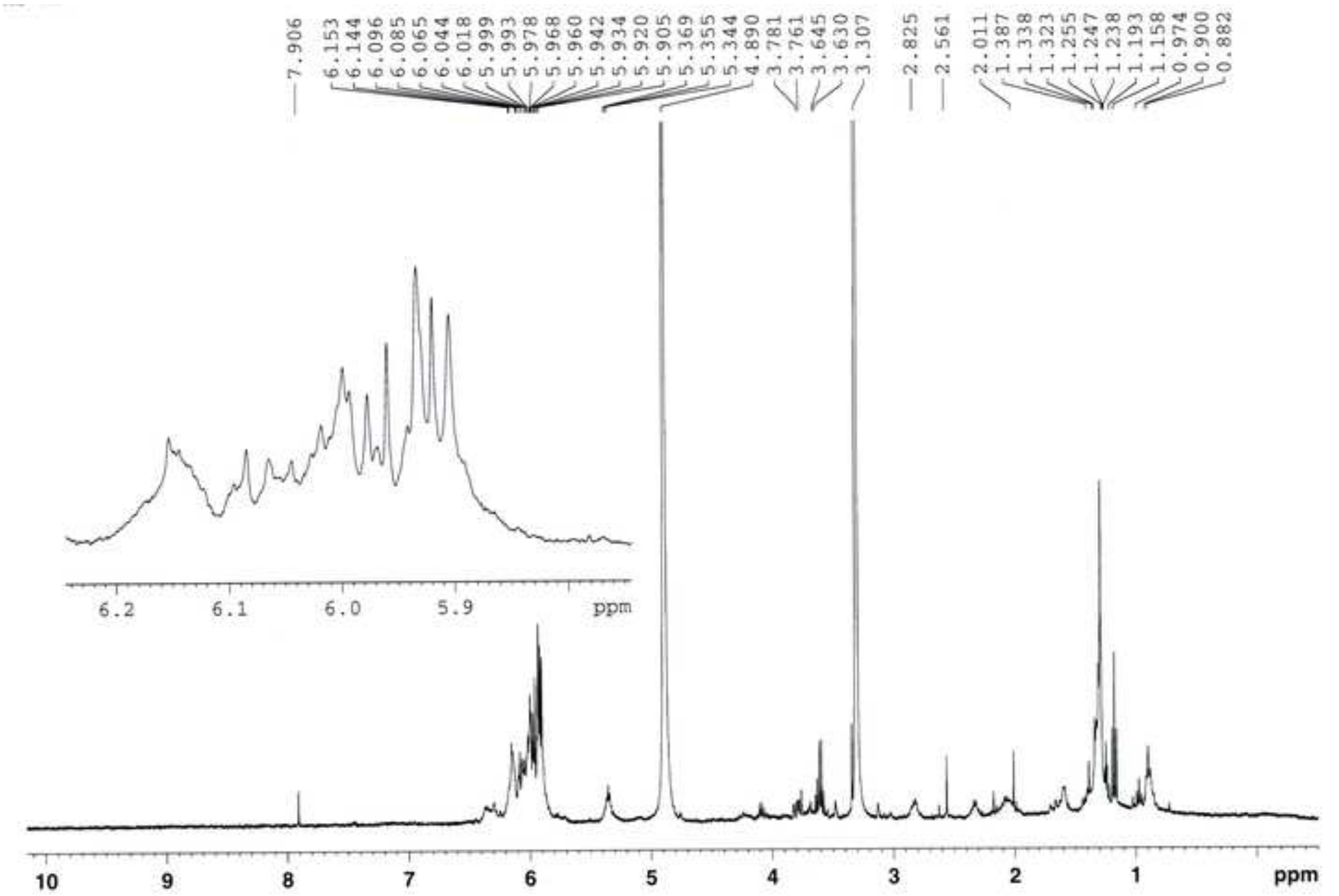
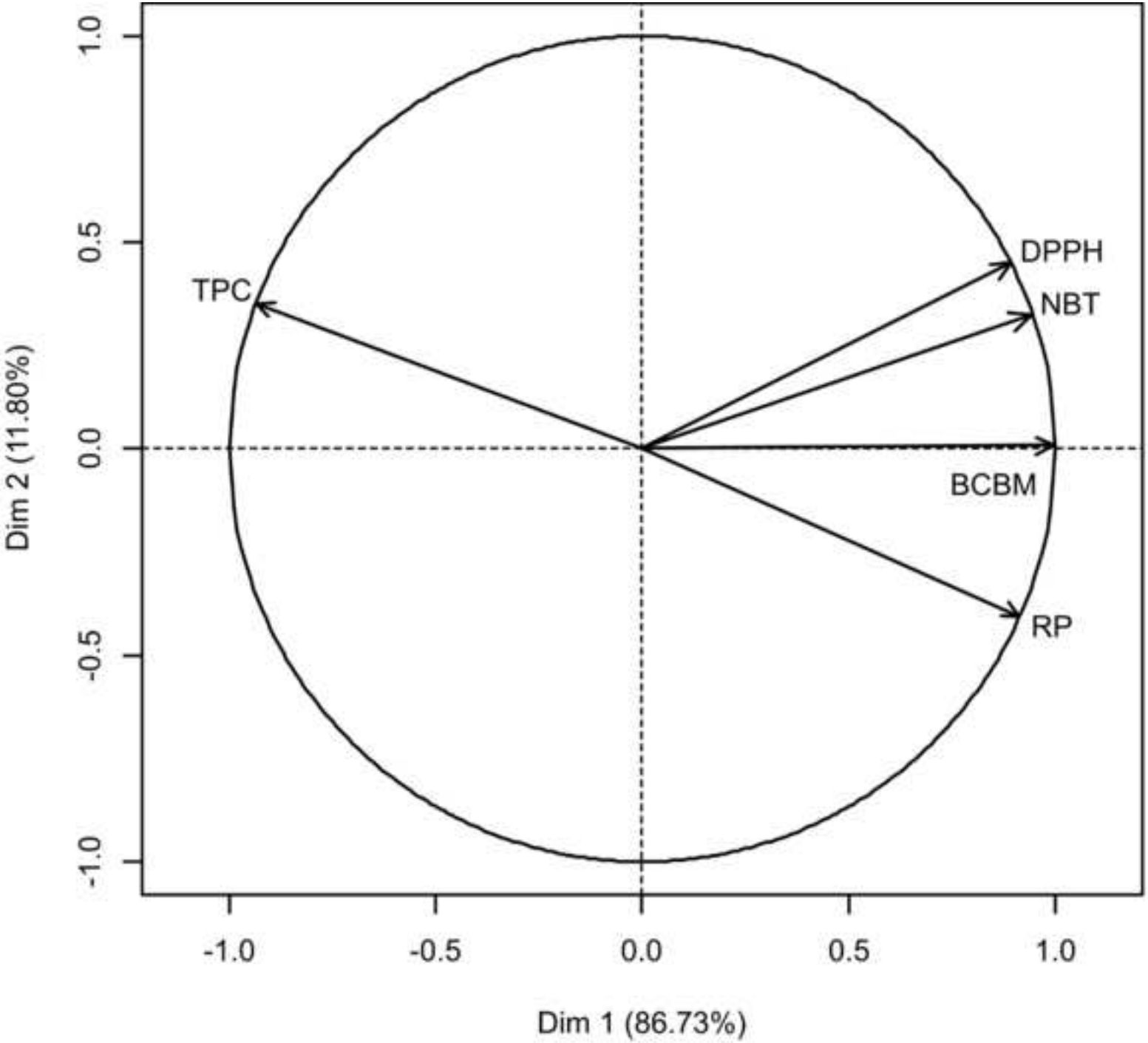


Figure 3

Click here to download line figure Fig3.tiff



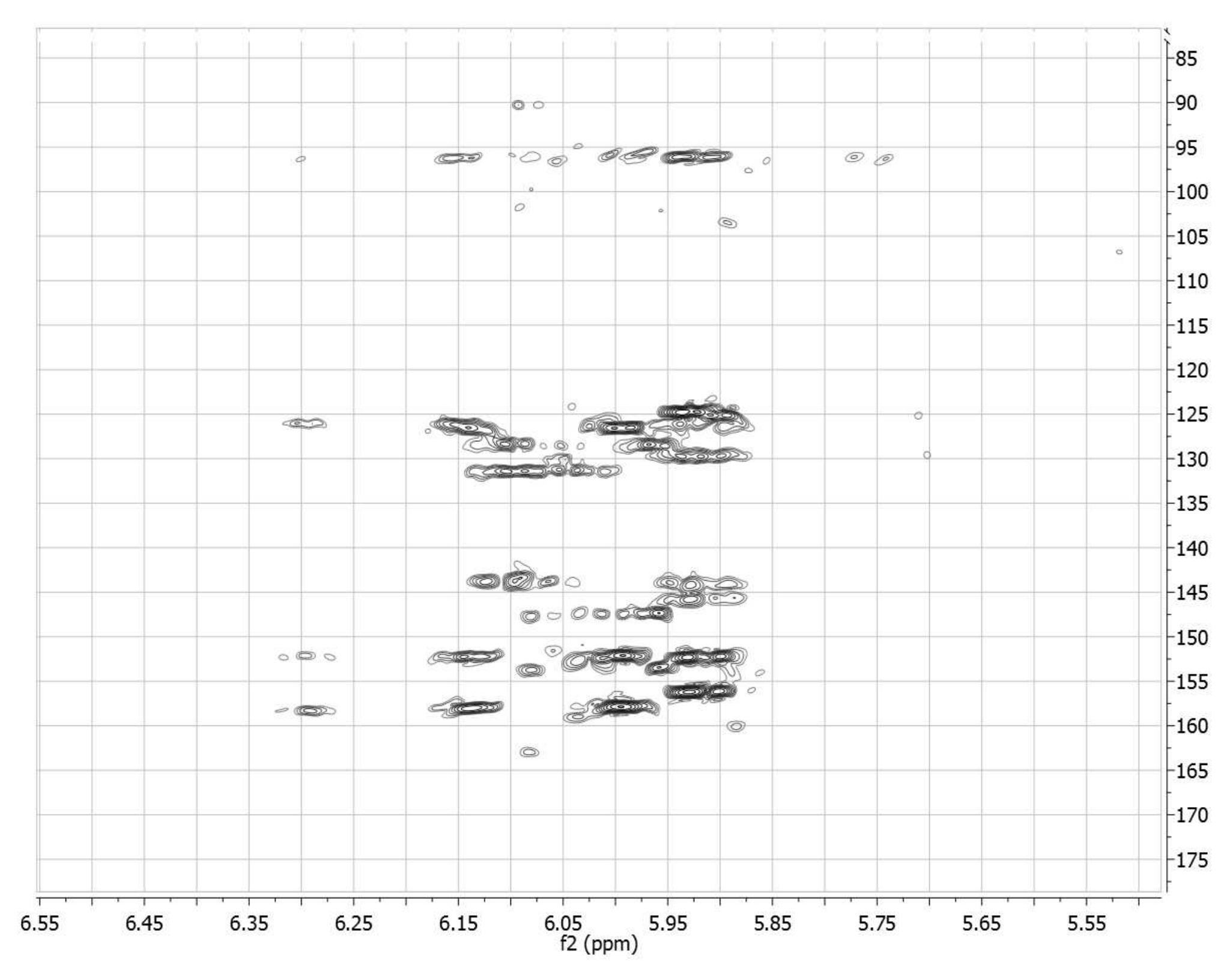


Figure 4

Click here to download line figure Fig4.eps

Figure 5

Click here to download line figure Fig5.eps

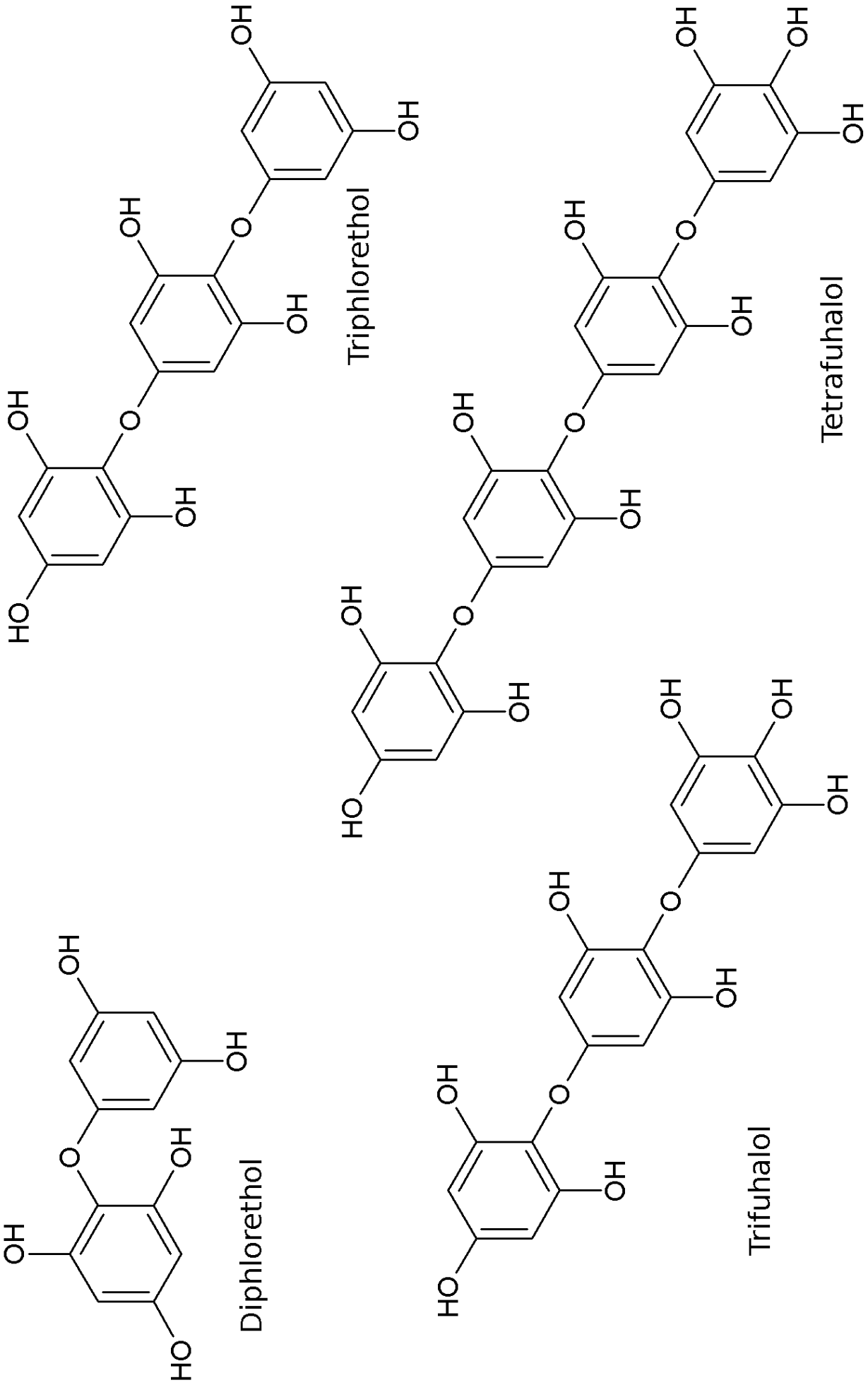


Table 1. Results of total phenolic content (TPC) and screening of several antioxidant activities measured on crude extract and fractions from *Halidrys siliquosa*, using radical-scavenging activity (DPPH; IC_{50}), reducing power assay (RP; EC_{50}), superoxide anion-scavenging test (NBT; IC_{50}), β-carotene bleaching method (BCBM, AAC_{700}), and ORAC value.

		TPC ($mg.g^{-1}$ DW)	DPPH; IC_{50} ($mg.mL^{-1}$)	RP; EC_{50} ($mg.mL^{-1}$)	NBT; IC_{50} ($mg.mL^{-1}$)	BCBM; AAC_{700} ($mg.mL^{-1}$)	ORAC value ($\mu molTE.mg^{-1}$)
	Samples		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Halidrys siliquosa	CE	182.47 ± 3.33 ^{b}	0.226 ± 0.032 ^{f}	0.618 ± 0.039 ^{e}	1.345 ± 0.273 ^{c}	1.007 ± 0.01 ^{f}	nd
	AqF	164.19 ± 7.58 ^{a}	1.002 ± 0.055 ^{e}	0.59 ± 0.014 ^{e}	2.441 ± 0.401 ^{d}	1.502 ± 0.077 ^{e}	nd
	EAF	975.73 ± 19.26 ^{c}	0.02 ± 3.54E10^{-4} ^{d}	0.058 ± 0.001 ^{a}	0.664 ± 0.019 ^{b}	0.206 ± 0.001 ^{d}	5.39 ± 1.08
Positive controls	BHA	nd	0.01 ± 4.82E10^{-5} ^{c}	0.074 ± 0.002 ^{b}	0.664 ± 0.022 ^{b}	0.015 ± 0.001 ^{c}	nd
	Trolox	nd	0.007 ± 3.40E10^{-4} ^{b}	0.404 ± 0.037 ^{d}	0.368 ± 0.042 ^{a}	0.007 ± 0 ^{b}	nd
	Ascorbic acid	nd	0.005 ± 8.06E10^{-4} ^{a}	0.088 ± 0.001 ^{c}	0.367 ± 0.045 ^{a}	nd	9.35 ± 0.63*
	α-tocopherol	nd	nd	nd	nd	0.005 ± 0 ^{a}	nd
	BHT	nd	nd	nd	nd	nd	24.1 ± 1.18*

Each value corresponds to the mean ± SD (n = 3). Significant differences determined by the Behrens-Fisher test ($p < 0.05$) are indicated by different letters. nd = not determined; CE = crude extract, AqF = aqueous fraction, EAF = ethyl acetate fraction, TE = trolox equivalent

* according to Huang et al. (2010).

Table 2. Antibacterial activity of *Halidrys siliquosa* ethyl acetate fraction (EAF, 5mg.mL^{-1} in 1% EtOH against three selected bacterial pathogens (*Pseudomonas aeruginosa*; *Staphylococcus aureus* and *Escherichia coli*). ETOH: Ethanol. Data are expressed in UFC.mL^{-1}

Bacterial strains		T0	T24h	Remark
Pseudomonas aeruginosa (ATCC 9027)	Growth control	1.1×10^6	1.3×10^9	
	1% EtOH	1.1×10^6	1.5×10^9	No effect
	EAF	1.1×10^6	<1	Bactericide activity
Staphylococcus aureus (ATCC 6538)	Growth control	1.9×10^6	2.2×10^9	
	1% EtOH	1.9×10^6	2.1×10^9	No effect
	EAF	1.9×10^6	7.5×10^1	Bactericide activity
Escherichia coli (ATCC 8739)	Growth control	1.6×10^6	1.5×10^9	
	1% EtOH	1.6×10^6	2.5×10^9	No effect
	EAF	1.6×10^6	<1	Bactericide activity

Table 3. HMBC assignments of the ethyl acetate fraction of *Halidrys siliquosa* (in MeOD)

	^{13}C (δ in ppm)	^{1}H (δ in ppm, *J* in Hz)
Quaternary methine groups	96.10	5.95, *s*
Diaryl-ether bond (ether linkage)	124.82	5.95, *s*
	125.07	5.92, *s*
	126.22	6.15, *s*
	126.52	6.14, *s*
	128.40	5.97, *s*
	129.74	5.92, *s*
	129.76	5.95, *s*
	131.21	6.05, *s*
	131.31	6.04, *s*
	131.41	6.10, *s*
Additional OH function (characteristic of fuhalol-type units)	143.80	6.12, *s*
	144.19	5.93, *s*
	145.77	5.93, *s*
	147.36	5.96, *d*, 2.5
Aromatic methine carbons (phenolic carbons)	152.26	6.14, *s*
	152.31	5.93, *s*
	152.66	6.03, *s*
	156.12	5.93, *s*
	156.21	5.2, *s*
	157.86	5.99, *s*
	158.04	6.14, *s*

Table 4. Experimental and calculated masses and putative chemical structures of polyphenolic oligomers identified by MALDI-TOF assisted by $^{13}C/^{1}H$ NMR, for the ethyl acetate fraction of *Halidrys siliquosa.*

Experimental measure (m/z)	Calculated m/z		Identified phloroglucinol oligomers	Calculated native masses (Da)
	M + Na (+ 22.99 Da)	M + H (+1 Da)		
273.06	273.19		(**1**) Diphlorethol	250.20
375.03		375.30	(**2**) Triphlorethol	374.30
413.29	413.29		(**3**) Trifuhalol	390.30
537.38	537.39		(**4**) Tetrafuhalol	514.40