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QUALITATIVE ASSESSEMENT OF POLYPHENOLS EXTRACTED FROM *FUCUS SPIRALIS* BIOMASS THROUGH HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

BY

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Abstract. Marine macroalgae biomass is a valuable resource of bioactive molecules and has received increased attention from the scientific community with the goal to generate high-tech bioproducts. Polyphenols represent one of these group of fine molecules that can be obtained from macroalgae. Quantitative as well as qualitative extracts are of utmost importance for the development of good macroalgal bioproducts. In this context, in order to contribute to the current undergoing research, the present study focuses on the assessment of polyphenols profile of extracts generated in an ultrasounds-assisted extraction from *Fucus spiralis* brown macroalgae. The qualitative analysis carried out through high-performance thin layer chromatography (HPTLC) aims to point out the presence of polyphenolic compounds from phenol-carboxylic acids to flavonoids and tannins.

Keywords: brown algae; polyphenols; ultrasounds-assisted extraction extracts; thin layer chromatography.

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1. Introduction

Polyphenols represent a diverse group of secondary metabolites with aromatic structure and key functionality in living organisms, having an important role in terrestrial, as well as in aquatic ecosystems, algae included (Ciupercă *et al*., 2019; Filote *et al*., 2021). Their bioactivity consists in antimicrobial, antioxidant, antitumor, antiallergenic and anti-inflammatory properties (Damaschin *et al*., 2018; Pătrăuțanu *et al*., 2020). Considering these valuable characteristics and in order to generate high-quality extracts with maximum yield, researches have focused on extraction and separation processes as well as on chemical profile assessment of polyphenols extracts. As far as the applied solvents are concerned, the main used are the organics (ethanol, acetone, methanol, etc.), water and mixtures of these in various ratios (Volf and Popa, 2018). The application of the emerging non-conventional extraction methods has also been more and more investigated for an efficient extraction of polyphenols (Yuan *et al*., 2018).

The crude extracts usually contain more than just one class of polyphenols. Flavonoids, bromophenols, phenolic acids, catechin, flavonol glycosides, as well as a characteristic polyphenolic group called phlorotannins can all be found in brown macroalgae (de Quirós *et al*., 2010; Yuan *et al*., 2018). Among this class of algae, *Fucus* and *Ascophyllum* are the species with the highest demonstrated amount of polyphenols, ranging between 1 and 14% (Agregán *et al*., 2018). In this regard, the reported antioxidant properties of *Fucus spp.* polyphenolic extracts makes them an alternative to the synthetic compounds that are used to conserve different food and cosmetic products (Catarino *et al*., 2019).

The current work aims i) to generate a polyphenolic extract from *Fucus spiralis* biomass applying a nonconventional green method, ultrasounds-assisted extraction, and ii) to assess its chemical profile using high-performance thin-layer chromatography (HPTLC).

2. Materials and Methods

2.1. Macroalgae Biomass Preparation

Fucus spiralis biomass was collected in February 2013 in Viana do Castelo, Northern Coast of Portugal. The algal biomass was washed with both tap and distilled water. Drying of the brown algae was performed in oven at 50 °C, for 24 hand afterwards, a soft grinding was applied in order to obtain particles with a size ≤ 0.5 cm. The pre-treated biomass was stored in a desiccator, in tightly sealed bags, for further use.

2.2. Extraction of Polyphenols

The crude polyphenolic extract was generated after an ultrasounds-assisted extraction process performed in an Elmasonic 120 ultrasonic bath following a protocol previously described (Lazăr *et al*., 2016). The extraction was carried out using ethanol 70% as solvent, a solid/liquid ratio of 1:10, at 40ºC and an extraction time of 45 min.

2.3. HPTLC Chromatographic Analysis

In the present study, a CAMAG thin-layer chromatography system (consisting of a LINOMAT IV applicator, a CAMAG documentation system with Canon digital video camera) was used. Also, Merck silica-gel HPTLC chromatographic plates, chromatographic purity solvents and standards were used. For HPTLC chromatographic impression of flavonoids and polyphenols, glycosylated flavonoids and aglycones as well as phenolic acids and phenol-carboxylic acids of HPTLC purity were used as standards. Other analytical reagents applied were methanol (99.98%, Chempur), ethyl acetate (purity ≥ 99.5%; SIGMA-ALDRICH), acetic acid (p.a., min. 99.0%), formic acid (98-100% p.a., Merck), NP (β-ethylamine diphenylborate) (min. 97%, Fluka) and PEG 400 (polyethylene glycol 400) (Fluka).

Before all determinations, the chromatographic plate was saturated for 30 min with the developer. Saturation and all determinations were performed at a temperature of 20-25°C.

In order to identify the target compounds, the *Rf* value and the colour or fluorescence of the sample spot were compared on the same chromatogram with the *Rf* value and the colour or fluorescence of the standard spot. The *Rf* value was determined according to equation:

$$Rf = \frac{a}{b} \tag{1}$$

where: *a* is the distance travelled by the compound from the point of application to the centre of the spot, [cm] and *b* – the distance travelled by the developer from the point of application to the front, passing through the centre of the spot, [cm].

In some cases, the identification of a compound could also be performed using the *Rr* value obtained using equation:

$$Rr = \frac{a}{c} \tag{2}$$

where: *a* is the distance travelled by the substance from the point of application to the centre of the spot, [cm] and c – the distance travelled by the reference substance from the point of application to the centre of the spot, [cm].

The samples and standard solutions were applied to chromatographic plate in bands using the LINOMAT IV applicator. Highlighting of spots on the chromatogram was performed by examining them as such or after treatment with suitable reagents, in visible (366 nm) or in ultraviolet (254 nm) light.

Identification can be done in two ways. The first option is to perform the process without a chemical treatment - in ultraviolet light at 254 nm, all flavonoids stop fluorescence and appear as black spots on the chromatoplate, while at 366 nm, yellow, blue or green fluorescent areas could appear. The second option is to treat the chromatoplates with identification reagents which results in intense fluorescence response in ultraviolet light.

HPTLC (plates silica gel-Merk) chromatographic plates, 60 F245 20x10 were used to perform the analysis. The plates were first washed in methanol and activated at 120°C for 20 min. The references compounds were prepared by dissolving in methanol an exactly weighed of standards (Table 1).

Table 1

Reference Substances Used in the HPTLC Qualitative Determinations Carried out on Brown Algae Extracts

Reference substance	Synonym/Molecular formula	Purity%, Provider
Investigation POLYPHENOLCARBOXYLIC ACIDS		
Clorogenic acid	3-(3,4-Dihydroxycinnamoyl) quinic acid / $C_{16}H_{18}O_9$	95%, Sigma-Aldrich
Neoclorogenic acid	*trans*-5-*O*-Caffeoylquinic acid/ $C_{16}H_{18}O_9$	98%, HPLC, Sigma-Aldrich
Ferulic acid	4-hidroxi-3-metoxicinamic acid/ $C_{10}H_{10}O_4$	99%, HPLC, Sigma-Aldrich
Gallic acid	3,4,5-Trihydroxybenzoic acid/$C_7H_6O_5$	95%, Fluka
p-cumaric acid	4-Hydroxycinnamic acid	98%, Fluka
Investigation FLAVONOIDS		
Rutin	Quercetin 3-rutinozida / $C_{27}H_{30}O_{16}$	95%, HPLCSigma-Aldrich
Hyperoside	Quercetin 3-D-galactoside / $C_{21}H_{20}O_{12}$	97%, HPLC, Sigma-Aldrich
Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, 3,3′,4′,5,6-Pentahydroxyflavone, Quercetin-3-O-rhamnoside / $C_{15}H_{10}O_7$	98%, HPLC, Sigma-Aldrich
Investigation TANNINS		

Catechin	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol / $C_{15}H_{14}O_6$	99%, HPLC, Fluka
Epicatechin	(−)-*cis*-3,3,4′,5,7-Pentahydroxyflavane, (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol/ $C_9H_8O_4$	Rotichrom TLC, Roth
Tannic acid	2,3-dihydroxy-5-({[(2R,3R,4S,5R,6R)-3,4,5,6-tetrakis({3,4-dihydroxy-5-[(3,4,5-trihydroxyphenyl)carbonyloxy]phenyl}carbonyloxy)oxan-2-yl]methoxy}carbonyl)phenyl 3,4,5-trihydroxybenzoate/ $C_{76}H_{52}O_{46}$	86%, ACS

For the **assessment of phenol-carboxylic acids** the samples and references were applied separately on the plate, in a maximum of 14 bands, the distance between the bands of 7 mm, the application volume 8 μL (for sample) and 5 μL (for standard), the band length of 14 mm and application speed of 8 s/μL.

The plates were developed in a saturated chamber, at room temperature (20-22°C), for 30 min using as mobile phase a mixture of ethyl acetate: formic acid: acetic acid: water = 20: 2.2: 2.2: 5.4 (v/v/v/v). After development, the plates were air dried at room temperature.

The appearance of a yellow, yellow-orange or yellow-green spot indicates the presence of flavonoid compounds while the appearance of blue-fluorescent spots indicates the phenol-carboxylic acids.

For **the tannins investigation**, the sample and standards were applied separately on plate, up to 14 bands, the distance between the strips being 10 mm, the application volume 16 μL and 6 μL (catechin), 7 μL (epicatechin) and 10 μL (tannic acid), the application volume 16 μL and application rate of 8 s/μL. The appearance of blue-gray spots indicates the presence of tannins.

3. Results and Discussion

Thin layer chromatography is a physic-chemical separation method, consisting of a stationary phase - chromatographic plates of glass or other materials on which a thin layer of adsorbent is applied (silica gel, kieselguhr, cellulose microcrystalline, etc.) and a mobile phase (developer) - a solvent or mixture of solvents - moving in a given direction. The method is based on a process of differentiated dynamic migration of a compound in a mixture due to differences in adsorption, molecular mass, solubility, etc.

Qualitative assessment of the polyphenolic profile of crude extract from *Fucus spiralis* brown alga, performed by thin layer chromatography, highlight

the appearance of blue/fluorescent blue bands, visualized at 366 nm, indicating the presence of polyphenols. Also, the appearance of yellow bands in the same chromatographic conditions point out the presence of flavonoids at different *Rf* values (Fig. 1).

HPTLC images clear indicated the separation of standards identified at the following *Rf* values: rutin - 0.42; chlorogenic acid - 0.51; neochlorogenic acid - 0.56; p-coumaric acid - 0.95; quercetin - 0.95; gallic acid - 0.90; ferulic acid - 0.93. Nevertheless, none of these standards have been identified in the crude extract by visualization at 366 nm (Fig. 1, Table 2). Also, the screening at 254 nm indicated that the applied standards were not assigned in any of the samples (Fig. 2).

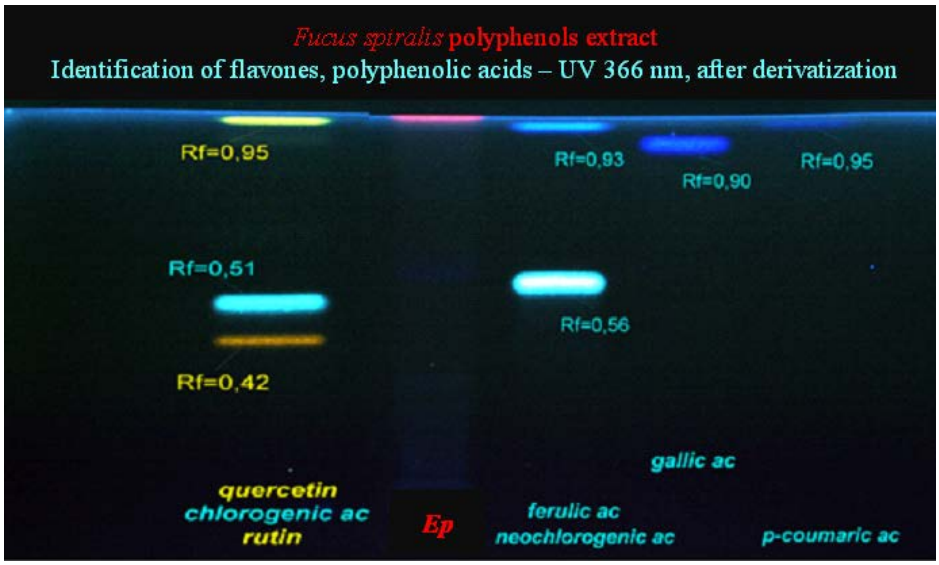
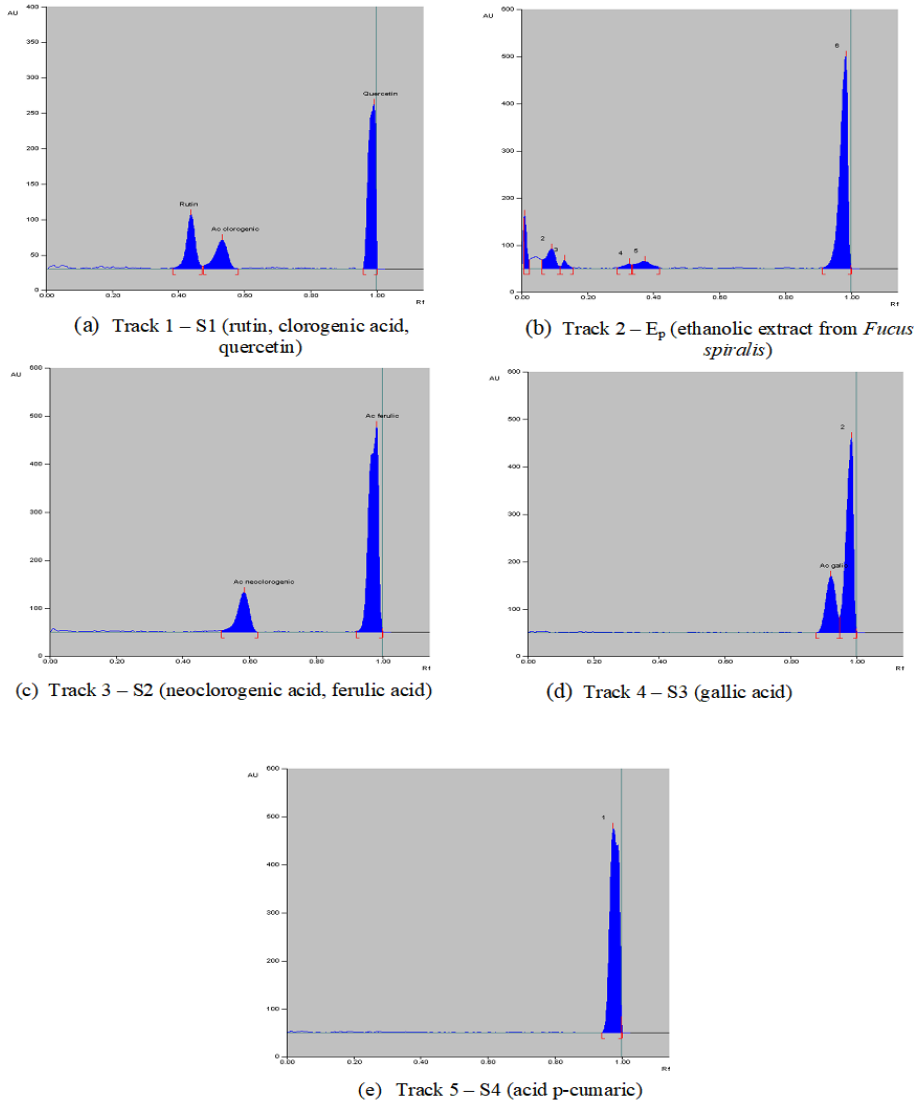


Fig. 1 – Identification chromatogram of flavonoids and polyphenols in brown algae extract, at 366 nm, after derivatization.
Tracks: 1 – S1 (rutin, clorogenic acid, quercetin); 2 – E_p (brown algae ethanolic extract); 3 – S2 (neoclorogenic acid, ferulic acid); 4 – S3 (gallic acid); 5 – S4 (p-cumaric acid).

Table 2
Rf Values Identified in the Fucus Spiralis Brown Algae Extract

	Identified constituents / Colour bands
Rf value	**E_p**
0.97	Distinct pink-purple band



(a) Track 1 – S1 (rutin, clorogenic acid, quercetin)

(b) Track 2 – E_p (ethanolic extract from *Fucus spiralis*)

(c) Track 3 – S2 (neoclorogenic acid, ferulic acid)

(d) Track 4 – S3 (gallic acid)

(e) Track 5 – S4 (acid p-cumaric)

Fig. 2 – Screening performed at 254 nm on *Fucus spiralis* crude extract.

However, the qualitative phytochemical study performed by thin layer chromatography showed that the extract obtained from brown alga *Fucus spiralis*, by an ultrasound extraction, using as solvent ethanol 70% v/v still has a profile for polyphenols and flavones (Fig. 2*b*). Only, the peak present in the chromatogram could not be identified based on the standards proposed in this study.

Chromatographic identification of tannins from 70% ethanolic extract of brown algae (Ep), obtained by ultrasound extraction was also performed, using as standards catechin (S1), epicatechin (S2) and tannic acid (S3), respectively.

The HPTLC image indicates the separation of standards and constituents from samples by blue-gray or green-gray coloured bands. The reference substances were identified at the following *Rf* values: catechin - 0.46; epicatechin - 0.42; tannic acid - 0.01. Considering only these commune standards and following the visualization in the visible range as well as spraying with the identification reagent (ferric chloride), the specific constituents of the crude extract could not be identified (Fig. 3).

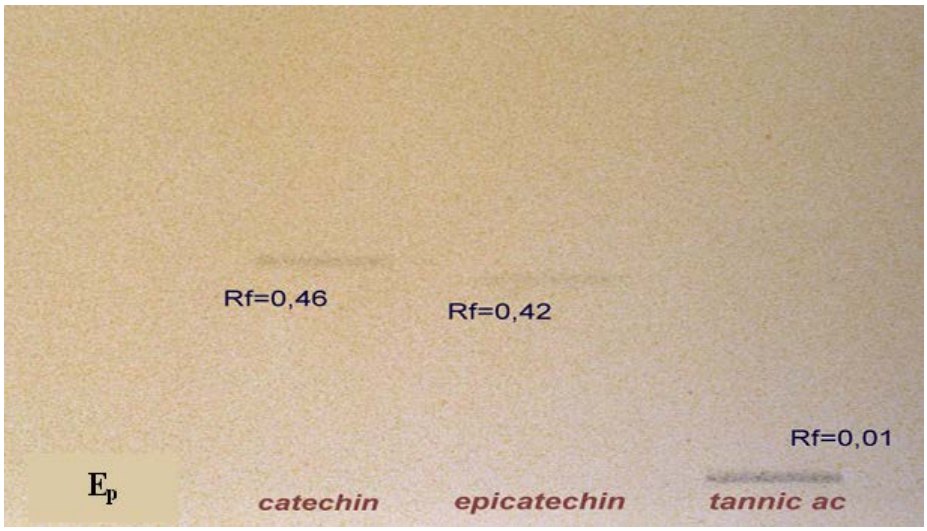


Fig. 3 – Tannin identification chromatogram in *Fucus spiralis* brown algae extract.

Tracks: 1 – E_p (ethanolic extract from brown algae); 2 – S1 (catechin); 3 – S2 (epicatechin); 4 – S3(tannic acid).

However, the screening at 254 nm performed for the quantitative evaluation of the compounds provided the following information: the catechin and epicatechin standards were not assigned in the sample while tannic acid was assigned (Fig. 4).

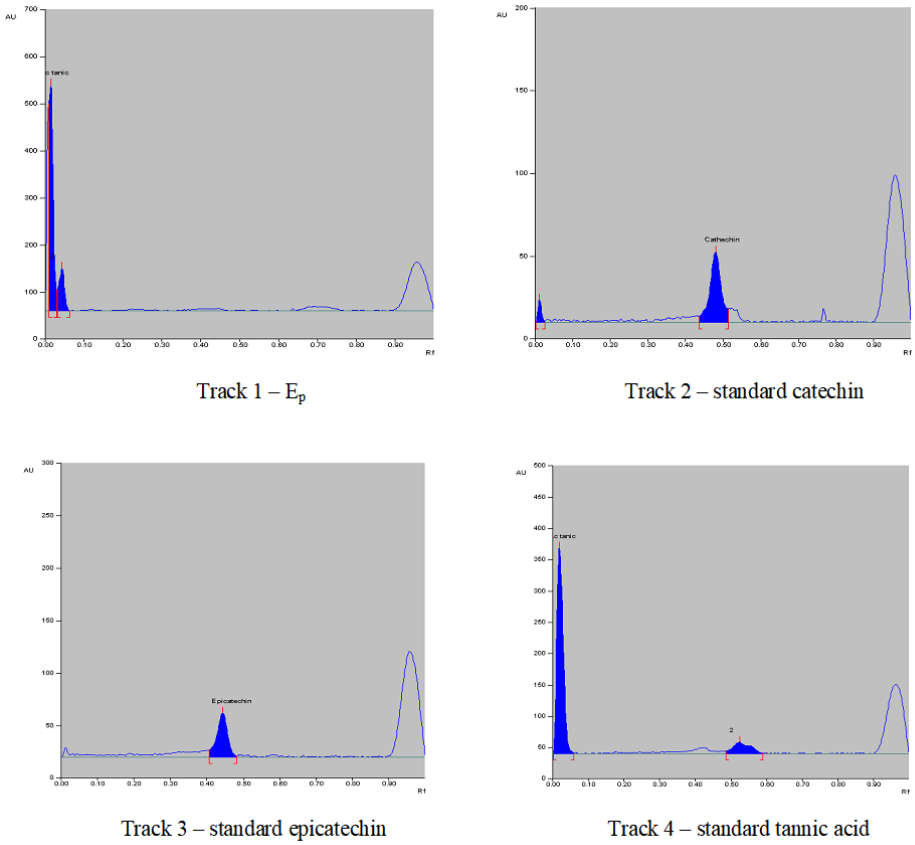


Fig. 4 – Identification of tannic acid in *Fucus spiralis* brown algae extract.

4. Conclusions

High-performance thin layer chromatography could be used as a qualitative identification method of natural valuable molecules such as polyphenols. However, in order to identify all targeted compounds, a wide range of standards must be used. Moreover, for specific feedstock such as macroalgae, very specific standards must be used.

The qualitative phytochemical study performed by thin layer chromatography showed that the extract obtained from brown alga *Fucus spiralis*, by an ultrasound extraction, using as solvent ethanol 70% v/v has a profile for phenol-carboxylic acid and flavones that could not be identified based on the standards proposed in this study. Also, the screening performed at 254 nm highlighted the presence of tannins.

In order the have a complete polyphenolic profile the HPTLC analysis must be resumed considering very specific standards such as phlorotannins or applying other method such high performance liquid chromatography coupled with mass spectrometry.

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ESTIMAREA CALITATIVĂ A COMPUȘILOR POLIFENOLICI DIN *FUCUS SPIRALIS* PRIN CROMATOGRAFIA DE ÎNALTĂ PERFORMANȚĂ PE STRAT SUBȚIRE

(Rezumat)

Biomasa macroalgelor marine este o resursă de molecule bioactive căreia i s-a acordat o atenție crescândă din partea comunității științifice cu scopul de a genera bioproduse. Compusii polifenolici reprezintă unul dintre grupurile de molecule valoroase care pot fi obținute din macroalge. Extractele cantitative, precum și cele calitative sunt de importanță majoră pentru dezvoltarea bioproduselor macroalgale bune. În acest context, prezentul studiu se concentrează pe analiza profilului polifenolic al extractelor generate prin extracție asistată de ultrasunete din macroalga brună *Fucus spiralis.* Analiza calitativă efectuată prin cromatografie de înaltă performanță pe strat subțire (HPTLC) poate evidenția prezența compușilor polifenolici, cu structură mai simplă sau mai complicată, de la acizi fenol-carboxilici la flavonoide și taninuri în condițiile în care compușii martor care s-au folosit în experimentare se regăsesc și în materialul vegetal extras. Analiza HPTLC realizată pentru extractul polifenolic din *Fucus spiralis* a infirmat prezența acizilor clorogenic, neoclorogenic, ferulic, galic și paracumaric dar și a unor flavonoide și taninuri precum rutinul, quercetina, catechina, epicatehina și acidul tanic. Analiza evidențiază însă prezența unor compuși polifenolici specifici acestei alge, probabil din clasa florotaninurilor.