Macroalga *Padina pavonica* water extracts obtained by pressurized liquid extraction and microwave-assisted extraction inhibit hyaluronidase activity as shown by capillary electrophoresis

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Key words: Enzyme kinetics, hyaluronidase, capillary electrophoresis, algae, electroporation.

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Abstract

Hyaluronidase degrades hyaluronic acid, the principal component of the extracellular matrix. Inhibition of this enzyme is thus expected to hinder skin aging. Brown alga *Padina pavonica* activity towards hyaluronidase was evaluated using capillary electrophoresis (CE)-based enzymatic assays. This green technique allows evaluation of the biological activity of the natural material in an economic manner. Pressurized liquid extraction (PLE), microwave assisted extraction (MAE), supercritical fluid extraction and electroporation extraction techniques were used. Extraction conditions were optimized to obtain cosmetically acceptable *Padina pavonica* extracts with the best inhibition activity. CE-based assays were conducted using only a few nanoliters of reactants, a capillary of 60 cm total length and of 50 μm internal diameter, +20 kV voltage for separation in 50 mM ammonium acetate buffer (pH 9.0) and 200 nm wavelength for detection. The reaction mixture was incubated for 1 h and CE analysis time was about 11 min. A novel online CE-assay using transverse diffusion of laminar flow profiles for in-capillary reactant mixing allowed efficient monitoring of hyaluronidase kinetics with K_m and $V_{\max}$ equal to $0.46 \pm 0.04 \text{ mg.mL}^{-1}$ and $137.1 \pm 0.3 \text{ nM.s}^{-1}$ ($r^2=0.99$; $n=3$), respectively. These values compared well with literature, which validates the assay. Water extracts obtained by PLE (60°C; 2 cycles) and MAE (60°C; 1000 W; 2 min) presented the highest anti-hyaluronidase activity. The half maximal effective concentration (IC_{50}) of water PLE extract was $0.04 \pm 0.01 \text{ mg.mL}^{-1}$ ($r^2=0.99$; $n=3$). This value is comparable to the one obtained for *Eisenia bicyclis* phlorotannin fractions ($\text{IC}_{50} = 0.03 \text{ mg.mL}^{-1}$), which makes *Padina pavonica* bioactivity very promising.

Highlights:

1. Development of online assay for hyaluronidase inhibition by CE
2. Activity of a brown alga, *Padina pavonica*, against hyaluronidase was studied
3. Different extraction techniques were used and optimized to obtain active extracts
4. IC_{50} of water and ethyl acetate extracts toward hyaluronidase evaluated by CE

1. Introduction

The extracellular matrix of the skin consists of a complex and dynamic network of macromolecules composed of a variety of proteins such as collagen and elastin as well as proteoglycans to which glycosaminoglycan (GAG) chains are attached. The most common GAG in the dermis is hyaluronic acid (HA) or hyaluronan [1,2]. Since it is extremely hydrophilic, HA has a high hydration capacity contributing to the viscoelastic properties of the skin. Hyaluronidase is the endoglycosidase enzyme that randomly cleaves internal β -N-acetyl-hexosamine and glucosidic linkages in hyaluronic acid (HA) [3,4]. In addition to its important role in skin aging, hyaluronidase has been successfully utilized in ophthalmic surgery and is now being implemented in dermatosurgery and other surgical disciplines [5]. Finding new effectors of this enzyme is of great interest. Shibata et al. [6] evaluated the effect of *Eisenia bicyclis* brown alga phlorotannins on hyaluronidase by using an *in vitro* assay. The 8,8'-bieckol, an eckol-type phlorotannin, was shown to have the strongest inhibitory effect with an IC_{50} of 0.03 mg.mL^{-1} . Valentao et al. [7] also found that the phlorotannin extracts from four brown seaweeds, particularly from *Fucus spiralis*, had anti-hyaluronidase activity with an IC_{50} equal to 0.73 mg.mL^{-1} . Moreover, Ratnasooriya et al. [8] showed that Sri Lankan black tea (*Camellia sinensis* L.) brew had a moderate anti-hyaluronidase activity ($IC_{50} = 1.09 \pm 0.12 \text{ mg.mL}^{-1}$).

Algae are rich in bioactive compounds which increase their importance nowadays in many fields [9-11]. In this work, *Padina pavonica* alga was studied for its inhibition activity towards hyaluronidase. This brown alga is commonly known as Peacocks tail and is found in the Atlantic Ocean and in the Mediterranean Sea. *Padina pavonica* alga is known for its capacity of restoring the calcium metabolism in the epidermis and maintaining a youthful aspect of the skin by promoting the renewal of GAGs, thereby boosting their protective action [12]. Moreover, Ktari and Guyot [13] evaluated the cytotoxic activity of *Padina pavonica* dichloromethane extracts against human buccal epidermal carcinoma cells and found that 0.01 mg.mL^{-1} dose was sufficient to obtain 100% inhibition of tumor cell survival. The antioxidant and antifungal activities of this alga were studied by Chbani et al. [14] after maceration and soxhlet extractions with methanol. Ethyl acetate fraction was adequately prepared and showed the best antifungal activity toward *Candida glabrata* and *Candida krusei* species. This activity was referred to phenolic compounds. Mohamed et al. [15] studied the antiviral activity of *Padina pavonica* against *herpes simplex virus* (HSV) and *hepatitis A virus* (HAV). The water

extract of sulfated heteropolysaccharide fraction showed an important antiviral activity (72-73%) at 0.02 mg.mL⁻¹.

Nowadays, different extraction techniques can be used. The conventional one such as soxhlet extraction is very time consuming (several hours) and requires relatively large quantities of solvent which cause environmental problems and high operating costs. Recently, several novel extraction techniques such as pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) as well as ultrasonic- and electroporation - assisted extractions have been developed [16-20]. PLE is an automated extraction technique developed by Richter et al. [21] in which elevated temperatures and pressures are applied to achieve extractions in short periods of time. MAE utilizes microwave energy for heating, and thus increasing the mass transfer rate of the solutes from the sample matrix into the solvent. The first extraction of a natural product by MAE was achieved by Pare in 1994 [22]. These techniques are widely used to extract biologically active molecules [23-25]. SFE is a promising green technology that can potentially displace the use of traditional organic solvents by carbon dioxide (CO₂) for extraction. CO₂ facilitates a safe extraction due to its low toxicity, low flammability and lack of reactivity [18,26]. SFE presents additional advantages such as operating at a low temperature that reduces the thermal degradation of labile compounds and high selectivity in the extraction of target compounds. Recently, a lab-made apparatus using electroporation-assisted extraction was developed in our group to extract amino acids from microalgae cells by applying a continuous voltage of few volts [20]. Electroporation is an efficient process for breaking cell membranes that was also used for extracting proteins and small molecules from bacterial and eukaryote cells [27,28].

In this study, *Padina pavonica* extraction was optimized using MAE, SFE, electroporation and PLE. Different parameters were studied to obtain the most efficient anti-hyaluronidase extract adapted for cosmetic use. Miniaturized CE enzymatic assays were chosen to study algae extract bioactivity [29,30]. Our previously developed pre-capillary CE-based hyaluronidase assay [31] and a novel online assay were conducted. The novelty of the online assay consists of studying the degradation of a multifunctional high molecular weight polysaccharide, the hyaluronic acid, into different poly(di)saccharide fragments. These fragments will be then considered as novel substrates of hyaluronidase inside the capillary [4]. Moreover, algae extracts must be homogeneously mixed with the enzyme and its substrate(s) in the capillary for online evaluation of the bioactivity of this vegetal. IC₅₀ value of the most efficient extracts was determined.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and used as received without further purification. Ammonium acetate ($C_2H_3O_2NH_4$, purity $\geq 98\%$), hyaluronidase type I-S from bovine testes (BTH, 400-1000 units.mg⁻¹ solid), sodium acetate (CH_3COONa , purity $\geq 99\%$), sodium hydroxide (NaOH, purity $\geq 98\%$) and oligohyaluronic acid 4 (oligo-HA4 or tetrasaccharide (**Tet**), $C_{28}H_{44}N_2O_{23}$) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Hyaluronic acid sodium salt (HA) from *streptococcus pyrogenes* was purchased from Merck Millipore (Molsheim, France). Glacial acetic acid (CH_3CO_2H), ammonia (NH₃, purity 28%), diethyl ether (Et₂O), ethanol (EtOH), ethyl acetate ($C_4H_8O_2$) and sulfuric acid (H₂SO₄, purity 96%) were purchased from VWR International (Fontenay-sous-Bois, France). Petroleum ether was purchased from Carlo Erba (Val-de-Reuil, France). The CO₂ was supplied by Linde (Munich, Germany). Ultra-pure water (18 MΩ.cm) was produced from an Elga apparatus (Elga, Villeurbanne, France). Syringes and hydrophilic polyvinylidenedifluoride (PVDF) Millex-HV Syringe Filters, pore size 0.45 μm, were purchased from Millipore (Molsheim, France).

2.2. Solutions

Solutions were filtered through PVDF Millex-HV Syringe Filter before use and stored at 4°C. The pH of the buffers was measured with a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France). The different buffers were prepared each day and their parameters were given by Phoebus software (Analisis, Namur, Belgium).

Sodium acetate at 2 mM was used as the incubation buffer (IB). Its pH was fixed at 4.0 by adding glacial acetic acid. The background electrolyte (BGE) was ammonium acetate 50 mM with a pH of 9.0 adjusted with ammonia.

Stock solutions of BTH, HA and **Tet** were prepared in the IB at a concentration of 2 mg.mL⁻¹ and diluted to the appropriate concentrations in the same buffer. The enzyme stock solution was prepared fresh every day.

When obtained, extracts were evaporated under a stream of nitrogen. Stock solutions of 1 mg.mL⁻¹ were obtained by dissolving the dried extracts in EtOH-water (10:90, v/v) except for water extracts which were dissolved in pure water. Each extract was then diluted in IB to the appropriate concentration and the incubation with hyaluronidase was done according to our previous work [31]. EtOH percentage did not exceed 2 % (v/v) in the final reaction mixture.

2.3. *Plant materials*

Brown seaweed *Padina pavonica* was manually collected from the coast of Berbara (also spelled Al-Barbara) in Byblos city in Lebanon at the end of May 2015. GPS coordinates of Berbara are 34° 11' 33" N 35° 38' 31" E. It has an average elevation of 200 meters above the Mediterranean sea level. The collected samples were transported to the laboratory where they were first cleaned and washed thoroughly with fresh water to remove salts, sand and epiphytes. The algae were lyophilized and ground to powder of about 100 µm size and stocked at ambient temperature.

2.4. *Capillary electrophoresis conditions*

The capillary electrophoresis system used was a PA800+ automated CE instrument from AB Sciex (Brea, CA, USA) equipped with a photodiode array detection system. The control of CE was performed using 32 Karat software (Beckman Coulter). All through this study, uncoated fused-silica capillaries (60 cm x 49.8 cm x 50 µm) purchased from Polymicro Technologies (Phoenix, AZ, USA) were used. New capillaries were initially conditioned by performing the following rinse cycles: 1 M NaOH (20 min), water (5 min) and BGE (10 min). Between two runs, the capillary was flushed with NaOH 1 M (1 min), water (0.5 min), and BGE (3 min). At the end of each working day, the capillary was rinsed with NaOH 1 M (10 min) and water (10 min) to ensure a good cleaning of the inner capillary surface, and stored overnight in water. All rinse cycles were carried out at 30 psi. Hydrodynamic injections were performed at the anodic side of the capillary and normal polarity mode was used with a separation voltage of +20 kV. Electrophoretic analyses were realized at 25°C in an ammonium acetate buffer (pH 9.0, ionic strength of 50 mM). The detection of the substrate (HA) and the products was performed at 200 nm (bandwidth 10 nm). The corrected-peak area (CPA) of an analyte represents the peak area, which has been corrected for migration time. The relative standard deviation (RSD) of the corrected peak-areas and of the migration times was used to assess intra-day and inter-day repeatabilities. The limit of quantification (LOQ) was calculated as the concentration corresponding to a signal-to-noise ratio of 10.

2.5. *Offline (pre-capillary) enzymatic assays*

Based on our previous work, bovine testicular hyaluronidase (BTH) was chosen and its activity was determined by following the formation of HA tetrasaccharide fractions by UV absorption [31]. The enzymatic reaction was conducted at 37°C in a microvial of the CE instrument auto-sampler in a total volume of 50 µL of IB. 5 µL of hyaluronidase (0.2 mg.mL⁻¹) were pre-incubated with *Padina pavonica* extract during 10 min. Then, a small volume (10

μL) of HA (0.4 mg.mL⁻¹, corresponding to the K_m value in the literature of 0.46 mg.mL⁻¹ [32]) was added to the mixture and the incubation time was 1 hour. The reaction was then stopped by heating during 10 min at 90°C. After cooling, 5 nL (0.5 psi for 5 sec) of the reaction mixture was introduced into the capillary. Blank assays, without the addition of the enzyme, the substrate and/or the extract were conducted before each set of CE analysis.

2.6. Online enzymatic assays

Transverse diffusion of laminar flow profiles (TDLFP) approach was used for in-capillary mixing of hyaluronidase with hyaluronic acid. After filling the capillary with the BGE, the reagents were introduced at the inlet side of the capillary as follows: IB (0.5 psi for 5 s; ~5 nL), BTH (0.5 psi for 4 s; ~4 nL), HA (~4 nL), BTH (~4 nL) and IB (0.5 psi for 15 s; ~15 nL). As for the offline assays, the incubation time was 1 h and a voltage of +20 kV was applied for separation. The order of the plugs was based on the diffusion coefficient (D_m) of each reagent. These coefficients were estimated using the Stokes-Einstein equation [32]:

$$D_m = \frac{K_B \cdot T}{\eta} \sqrt{\frac{d N_A}{162 \pi^2 M}} \quad (1)$$

where D_m is the diffusion coefficient (cm².s⁻¹); K_B is the Boltzman constant (m².g.s⁻².K⁻¹); T is the temperature (Kelvin); η is the viscosity (m².s⁻¹); d is the density (kg.cm⁻³); N_A is the Avogadro constant (mol⁻¹) and M is the molecular weight of each reactant (kg.mol⁻¹).

According to equation (1), the following results were obtained: D_m (BTH) = 1.4x10⁻⁶ cm².s⁻¹ and D_m (HA) = 3.4x10⁻⁷ cm².s⁻¹.

For online inhibition assays, TDLFP sequence was adapted in order to inject a plug of extract between the enzyme and the substrate as follows: IB (~5 nL), BTH (~4 nL), extract (~4 nL), HA (~4 nL), extract (~4 nL), BTH (~4 nL) and IB (~15 nL). This sequence was chosen after several experimental assays since extract molecular diffusion cannot be predicted.

2.7. Kinetic analysis of hyaluronidase reaction - determination of V_{max}, K_m and IC₅₀

For online assays, BTH was incubated with a range of concentrations of HA including the K_m value reported in the literature (K_m = 0.46 mg.mL⁻¹ [32]). Thus, the concentrations of HA ranged from 0.10 to 1.2 mg.mL⁻¹. Each point was repeated three times (n=3). The initial reaction rate V_i (nM.s⁻¹) was calculated as the ratio of the substrate formed *per* time interval. The nonlinear curve-fitting program PRISM[®] 5.04 (GraphPad, San Diego, CA, USA) was used to determine V_{max} and K_m of each substrate according to the following equation:

$$V_i = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (2)$$

where V_i is the reaction rate (nM.s^{-1}), K_m is the Michaelis-Menten constant (mg.mL^{-1}), $V_{\max}$ is the maximum reaction velocity (nM.s^{-1}) and $[S]$ is the substrate concentration (mg.mL^{-1}).

Hydrolysis activity of BTH in the absence of the tested extracts was defined as 100%. The half maximal effective concentration (IC_{50}) was determined as the inhibitor (extract) concentration at which half of the difference between the maximum and the minimum enzymatic activity was obtained. The inhibition concentration at 50% was determined by plotting the percentage of inhibition (I%) versus log [extract]. The inhibition percentage (I%) for each extract was determined using the following equation:

$$I = \left(1 - \frac{A_x}{A_0}\right) \times 100 \quad (3)$$

where A_x is the CPA of the product (Tetrasaccharide or **Tet**) formed in the presence of extract and A_0 is the CPA of **Tet** formed in the absence of extract.

2.8. MAE

650 mg of the alga was placed in Teflon bombs in a microwave-assisted (MW) synthesis labstation (Start synth, Milestone, 175 Bergamo). Four solvents (petroleum ether, ethanol, ethyl acetate and water) were tested at 60°C and 1000W to evaluate the influence of solvent nature and polarity on *Padina pavonica* extraction and its effect on hyaluronidase activity. The choice of these solvents is based on previous studies [14,33-37]. Two additional extraction temperatures (Table 1) were tested using ethyl acetate (40°C and 80°C) or water (100°C and 120°C) at two different extraction times (2 and 5 min). After extraction, the resulting supernatant was considered as 100% algal liquid extract.

2.9. SFE

The SFE apparatus was a Sepharex SFE-500 apparatus provided by Jasco equipment (Tokyo, Japan). Stainless steel cylindrical extraction cells located in the oven with a 5 mL-capacity were used. The extraction cell was filled with 500 mg of *Padina pavonica* alga. The temperature was fixed at 30°C and the pressure at 15 MPa. Three different extractions were performed with a new sample of alga each time and lasted for 15 or 30 min with a flow rate of 3 mL.min^{-1} (Table 2). The co-solvent percentages in carbon dioxide were investigated as follows: CO_2 -EtOH (80/20 or 95/5, v/v) and CO_2 -EtOH-water (80/16/4, v/v/v).

2.10. Electrochemical / Electroporation extraction

A lab-made device already developed by our group was used for amino acid extraction studies [20]. It consisted of two parallel platinum electrode wires, each one having a surface area of 0.6 cm² (1 cm length x 0.1 cm radius), connected to the positive and negative outlets of a DC (AL841B 30VA – ELC, Annecy, France). The inter-electrode gap was 1.7-1.8 cm. 50 mg of *Padina pavonica* alga was suspended in 20 mL of distilled water (2.5 g.L⁻¹) and placed in a glass vessel, on a magnetic stirrer. The sample was stirred constantly to prevent cells from settling out of suspension over the time-course of extraction and to maintain steady the diffusion around the electrodes. Extraction was performed by applying a voltage (6, 12, 24 and 32 V) to the suspension at different duration times [20] (Table 3).

2.11. PLE

PLE was performed with an ASE 100 system from Dionex (Voisins le Bretonneux, France) equipped with 5 mL stainless steel cells. 650 mg of alga were mixed homogeneously with the same weight of diatomaceous earth and placed into the extraction cell. The extraction cells were placed into the PLE system and the extraction conditions were selected as follows: a static time of 10 min, followed by a flush elution with 65% volume and a nitrogen purge of 60 s with 2 cycles of extraction. The pressure was fixed at 150 bars. Different parameters were optimized to obtain the most efficient anti-hyaluronidase extract. Then, ethyl acetate, ethanol, petroleum ether, and water were tested as extraction solvents at 60°C. Extraction temperature and cycle number effect were evaluated for the optimal extraction solvent.

2.12. HPTLC analysis

HPTLC analyses were done on 10 × 20 cm HPTLC plates (Merck, Germany) by a CAMAG (Muttens, Switzerland) ATS4 Automatic TLC Sampler controlled by Win-CATS software and were observed with a Reprostar 3 illumination unit. 10 μ L of extract solutions and 4 μ L of standard solutions each at 1 mg.mL⁻¹ were laid down. Plate elution was performed in a horizontal developing chamber. After development, the plate was dried and observed under 254 and 366 nm light. The plate was then sprayed with different specific or non-specific reagents. For each compound family, elution mixture, visualization reagent and standards are mentioned in Table 4.

3. Results and discussion

Different extraction (MAE, SFE, electroporation and SFE) techniques were used to obtain the most efficient anti-hyaluronidase *Padina pavonica* extract for potential cosmetic usage. Several parameters were carefully optimized such as solvent nature, extraction time

and temperature, and number of cycles. Table 5 summarizes optimum conditions for the different extraction techniques as well as hyaluronidase inhibition results for comparison.

The extracts obtained were evaporated, reconstituted into an appropriate solvent (containing at least 90% water) and then diluted in the IB. When performing *in vitro* or *in cellulo* tests, it is impossible to use a pure solvent such as EtOH, DMSO or petroleum ether since these solvents are toxic for enzymes and cells [30,38]. The corresponding solutions were tested for their anti-hyaluronidase activity by offline CE-based enzymatic assay [31]. The activity of the most efficient extracts was also evaluated by an economic novel online CE assay. Whether for the offline or the online CE hyaluronidase assay, the degradation of the hyaluronic acid was monitored by quantifying the **Tet** fraction produced after electrophoretic separation and UV absorption at 200 nm (for more details see reference [31]).

3.1. MAE- optimization

MAE is a suitable technique for alga extraction [39,40]. *Padina pavonica* extraction conditions were optimized in terms of extraction solvent, temperature and time. Best inhibition results were obtained using an extraction time of 2 min with ethyl acetate at 40°C or with water at 60°C (electropherograms in Fig 1.a). The extraction temperature showed a higher influence on hyaluronidase inhibition rather than the extraction time (Table 1). Indeed, an increase of temperature is expected to improve compound solubility, diffusion rate and mass transfer in the solvent of which viscosity and surface tension are decreased. Fig.1.a shows that no products were detected compared to the blank assay (in the absence of an inhibitor) in the presence of 0.2 mg.mL⁻¹ water extract (Table 5) which corresponds to a 100% inhibition of hyaluronidase (equation 3). This result is very interesting since water is a green solvent suitable for cosmetic applications. Moreover, this indicates that *Padina pavonica* anti-hyaluronidase bioactive compounds are mostly polar.

3.2. SFE optimization

SFE has been widely applied to extract natural compounds from several vegetables [41,42]. CO₂ presents low viscosity, relatively high diffusivity rate and an easy diffusion through solid materials, which is expected to allow better transport properties than liquids and thus higher extraction yields. Another feature of this technique is that CO₂ is a gas at room temperature and atmospheric pressure. Therefore, the extract obtained is almost free of solvent. Because of its low polarity, CO₂ is less effective to extract polar compounds in natural matrices. For this, SFE extractions were performed in this study with three different solvent mixtures where the highly polar solvents, EtOH and water, were added between 5 and 20% of the mixture volume

to bring substantial changes on the solvation properties of supercritical CO₂. Two extraction durations, 15 and 30 min, were tested. Hyaluronidase inhibition results are reported in Table 2. It can be seen that in the presence of water as CO₂ modifier, even at only 4%, hyaluronidase was almost completely inhibited by 0.2 mg.mL⁻¹ of extract; I = 90% (Tables 2 & 5 and Fig.1.b). These results confirm those obtained in the previous section using MAE technique.

3.3. *Electroporation extraction optimization*

Electroporation has been recently developed by our group [20] as a simple, non-expensive and efficient extraction procedure for *Dunaliella Salina* microalga. The efficiency of this technique depends on the applied voltage during a certain time. Water is used as the extraction solvent which is, therefore, suitable for the extraction of polar molecules and for cosmetic applications. Different direct voltages were first applied (6, 12, 24 and 32 V) with 2 min of extraction time as mentioned in Table 3. In the first assays, NaCl was added to the suspension mixture to maintain a constant current between the different extraction experiments. Hyaluronidase was activated by the obtained extracts, which was attributed to the presence of NaCl reported elsewhere as an activator of this enzyme [3]. In the absence of additional NaCl, CE inhibition results showed that a voltage of 32 V applied during 2 min was the optimum for extracting molecules responsible for anti-hyaluronidase activity; I = 53% at 0.2 mg.mL⁻¹ (Tables 3 & 5). At this voltage, increasing the extraction time to 5 and 10 min allowed a relatively small improvement of the inhibition to 65% (Fig 1.c). Water extracts obtained by electroporation are therefore less efficient than those obtained by MAE and SFE.

3.4. *PLE optimization and extract analysis by HPTLC*

Based on the results obtained above, PLE experiments were first conducted using two extraction cycles at 60°C and the same four solvents tested with MAE (section 2.8). The corresponding electropherograms obtained by CE-offline assay for the inhibition of hyaluronidase at 0.2 mg.mL⁻¹ are shown in Fig 1.d. As for MAE, ethyl acetate and water extracts were found to be the most efficient with 92% and 100% inhibition, respectively (Table 5). Working at a higher extraction temperature of 80°C had little influence on inhibition results. At 40°C, ethyl acetate extracts showed only slightly better inhibition activity (I = 95%) whereas lower inhibition activity was noticed for water extracts (I = 90%). IC₅₀ values for PLE ethyl acetate (Fig 2.I) and water (Fig 2.II) extracts were evaluated using offline CE-assay [31] as 0.25 ± 0.01 mg.mL⁻¹ and 0.03 ± 0.01 mg.mL⁻¹, respectively. A better inhibition was obtained using *Padina pavonica* water extracts.

To our knowledge, no work emphasizing the anti-hyaluronidase effect of *Padina pavonica* is described in the literature. Ethyl acetate and water PLE extracts were analyzed by HPTLC to obtain information about their chemical content (Table 4). Several families of natural compounds (terpenes, flavonoids and amino acids) were absent in ethyl acetate and water extracts. On the other hand, polyphenols and lipids were only revealed in ethyl acetate extracts. Indeed, several studies mentioned [14,15] that this solvent mainly extracts phenolic compounds from *Padina pavonica* brown alga whereas water mainly extracts sulfated polysaccharides. The latter compounds are known for their anti-hyaluronidase activity [31,43,44] as well as for their antiviral and antioxidant properties [15,45]. Girish et al. [46] listed the different chemical classes of hyaluronidase inhibitors. Alkaloids, glycosaminoglycans, glycosides as well as oligo- and poly- saccharides are the main polar inhibitors. Further chemical characterization of the active extracts is needed to identify the family or even the molecule(s) responsible for hyaluronidase inhibition.

3.5. Hyaluronidase online kinetic study

CE has become a frequently used tool for miniaturizing enzyme assays using online mode mainly due to its well-recognized low sample consumption [47]. In this study, an online CE assay for monitoring hyaluronidase reaction was developed based on TDLFP for reactant in-capillary mixing (see experimental section). Hyaluronidase, having a lower diffusion coefficient ($D_m(\text{BTH}) = 1.4 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$) was injected first, followed by hyaluronic acid ($D_m(\text{HA}) = 3.4 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$). To improve the mixing of the enzyme with the substrate, BTH was injected between two plugs of HA which corresponds to the so-called sandwich mode [48]. A plug of IB was injected at the beginning of the injection sequence to prevent inactivating the hyaluronidase by the BGE (partial filling mode) [49]. This IB plug must have a rectangular shape to avoid its transversal diffusion, so its injection must occur for a relatively long time (5 s). A plug of IB was also injected (0.5 psi for 15 s) at the end of the injection sequence to improve overlapping of reactant plugs by increasing the length of longitudinal plug interfaces [50]. In this sequence, two plugs of BTH, one plug of HA and one plug of IB were diffusing axially through the parabolic injection zones occupying then a final volume that is certainly higher than their initial injection zone [51]. BTH and HA concentrations are thus expected to be diluted inside the capillary by a factor of 2/3 and 3, respectively.

When TDLFP was conducted in these conditions, and when a suitable incubation time was used (60 min), a quantifiable peak of the product **Tet** was obtained inside the capillary. V_{max} and K_m of BTH reaction were thus evaluated as $137.1 \pm 0.3 \text{ nM} \cdot \text{s}^{-1}$ and $0.46 \pm 0.04 \text{ mg} \cdot \text{mL}^{-1}$

($r^2 = 0.94$; $n=3$), respectively. The values were comparable to those reported in literature [32] using a spectrophotometric assay ($V_{\max} = 126 \text{ nM.s}^{-1}$ and $K_m = 0.46 \text{ mg.mL}^{-1}$) and to those obtained by the offline CE-UV/HRMS assay ($V_{\max} = 155.7 \pm 0.2 \text{ nM.s}^{-1}$ and $K_m = 0.49 \pm 0.02 \text{ mg.mL}^{-1}$; $n=3$), which validates the developed online assay.

Hyaluronidase inhibition by PLE *Padina pavonica* extracts was then monitored. A plug of the extract was injected between the enzyme and its substrate from both sides in the sandwich mode [52]. To determine the efficiency of this assay, *Padina pavonica* extract obtained by PLE under optimum conditions (water; 60°C ; 2 cycles) was used and its IC_{50} value was determined. Different concentrations of the PLE extract were injected in the TDLFP sequence. With the increase of extract concentration, the CPA of the tetrasaccharide decreased, showing that the anti-hyaluronidase effect of PLE extracts is dose-dependent. IC_{50} was found to be $0.04 \pm 0.01 \text{ mg.mL}^{-1}$; $r^2 = 0.99$ (Fig 2 II.b.2). This value is comparable to the one previously obtained by the offline assay. Good intra-day ($n=3$) and inter-day ($n=2$) repeatabilities were obtained with RSDs on the CPAs and on the migration times of **Tet** inferior to 2.8%. High sensitivity was obtained with a LOQ of $17 \mu\text{M}$ for **Tet**.

4. Conclusion

Padina pavonica was studied to obtain efficient and cosmetically acceptable anti-hyaluronidase extracts for use as anti-age skin products. A green analysis system and enzymatic assay based on CE were used to screen the bioactivity of the obtained extracts. The study of algal activity was not yet done by online CE-based assays which was shown to be a very efficient and particularly economic tool (few nanoliters of reactants). The *Padina pavonica* water extract obtained by PLE (water; 60°C ; 2 cycles) and MAE (water; 60°C ; 1000 W; 2 min) yielded the most potent inhibition. Its IC_{50} value was found to be of few tens of micrograms *per* milliliter, ten times lower than most of the efficient crude extracts described in the literature to our knowledge. Later on, this extract will be fractioned to define the relationship between the concentration of the major compounds present in the active extracts and the hyaluronidase activity and on the other hand to identify and to isolate the active molecule(s). Moreover, this alga water extract can also be useful in cancer therapy since many tumors overexpress hyaluronic acid and collagen [53].

Acknowledgements

429 Authors gratefully acknowledge Dr. Philippe Bernard (GreenPharma, France), Dr. Patrice
430 André (Botanicosm'ethic, France) and Dr. Christophe Masson (Cosmetic Valley, France) for
431 their support. The authors would especially like to thank Dr. Régis Delatouche (ICOA) and
432 Dr. Johanna Duval (ICOA) for their essential contribution to the use of the microwave
433 apparatus and the supercritical fluid extractor, respectively. Authors also would like to thank
434 the Region Centre (France) for its financial support (research project PODOUCE, APR 2014).
435 *The authors declare that there are no conflicts of interest.*

References

- [1] J. Necas, L. Bartosikova, P. Brauner, J. Kolar, Hyaluronic acid (hyaluronan): a review, *Vet. Med.* 8 (2008) 397-411.
- [2] E. Papakonstantinou, M. Roth, G. Karakiulakis, Hyaluronic acid: A key molecule in skin aging, *Dermatoendocrinol.* 4 (2012) 253-258.
- [3] R. Stern, M.J. Jedrzejewski, Hyaluronidases: their genomics, structures, and mechanisms of action, *Chem. Rev.* 106 (2006) 818-839.
- [4] S. Highsmith, J.H. Garvin, D.M. Chipman, Mechanism of action of bovine testicular hyaluronidase, *J. Biol. Chem.* 250 (1975) 7473-7480.
- [5] B.A. Buhren, H. Schrupf, N.-P. Hoff, E. Bölke, S. Hilton, P.A. Gerber, Hyaluronidase: from clinical applications to molecular and cellular mechanisms, *Eur. J. Med. Res.* 21 (2016) 1-7.
- [6] T. Shibata, K. Fujimoto, K. Nagayama, K. Yamaguchi, T. Nakamura, Inhibitory activity of brown algal phlorotannins against hyaluronidase, *Int. J. Food Sci. Tech.* 37 (2002) 703-709.
- [7] F. Ferreres, G. Lopes, A. Gil-Izquierdo, P.B. Andrade, C. Sousa, T. Mouga, P. Valentão, Phlorotannin extracts from fucalae characterized by HPLC-DAD-ESI-MSn: approaches to hyaluronidase inhibitory capacity and antioxidant properties, *Mar. drugs* 10 (2012) 2766-2781.
- [8] W.D. Ratnasooriya, W.P.K.M. Abeysekera, C.T.D. Ratnasooriya, In vitro anti-hyaluronidase activity of Sri Lankan low grown orthodox orange pekoe grade black tea (*Camellia sinensis* L.), *Asian Pac. J. Trop. Biomed.* 4 (2014) 959-963.
- [9] K.A. Shams, N.S. Abdel-Azim, I.A. Saleh, M.-E.F. Hegazy, M.M. El-Missiry, F.M. Hammouda, Green technology: Economically and environmentally innovative methods for extraction of medicinal & aromatic plants (MAP) in Egypt *J. Chem. Pharm. Res.* 7 (2015) 1050-1074.
- [10] R. Kacem, Phenolic compounds from medicinal plants as natural anti-elastase products for the therapy of pulmonary emphysema, *J. Med. Plant Res.* 7 (2013) 3499-3507.
- [11] I. Wijesekara, R. Pangestuti, S.-K. Kim, Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae, *Carbohydr. Polym.* 84 (2011) 14-21.
- [12] D. Rivièrre, S. Roche, G. Rochefort, *Padina pavonica* (Linnaeus) Thivy, *Padine queue de paon*, DORIS (2016) <http://doris.ffessm.fr/ref/specie/1337>.
- [13] L. Ktari, M. Guyot, A cytotoxic oxysterol from the marine alga *Padina pavonica* (L.) Thivy, *J. Appl. Phycol.* 11 (1999) 511-513.
- [14] A. Chbani, N. Khaled, M. Hiba, Antioxidant and antifungal activities of *Padina pavonica* and *Sargassum vulgare* from the Lebanese Mediterranean Coast, *Adv. Environ. Biol.* 6 (2012) 42-48.
- [15] S.F. Mohamed, F.A. Agili, Antiviral sulphated polysaccharide from brown algae *Padina pavonica* characterization and structure elucidation, *Int. J. Chem.Tech. Res.* 5 (2013) 1469-1476.
- [16] G.J. Gil-Chavez, J.A. Villa, J.F. Ayala-Zavala, J.B. Heredia, D. Sepulveda, E.M. Yahia, G.A. Gonzalez-Aguilar, Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: an overview, *Compr. Rev. Food Sci. Food Saf.* 12 (2013) 5-23.
- [17] M. Bimakr, R.A. Rahman, F.S. Taip, A. Ganjloo, L.M. Salleh, J. Selamat, A. Hamid, I.S.M. Zaidul, Comparison of different extraction methods for the extraction of major

- bioactive flavonoid compounds from spearmint (*mentha spicata* L.) leaves, *Food Bioprod. Process.* 89 (2011) 67-72.
- [18] M.S.T. Barroso, G.V. nueva, A.M. Lucas, G.P. Perez, R.M.F. Vargas, G.W. Brun, E. Cassel, Supercritical fluid extraction of volatile and non-volatile compounds from *Schinus molle* L. , *Braz. J. Chem. Eng.* 28 (2011) 305-312.
- [19] J.C. Weaver, Y.A. Chizmadzhev, Theory of electroporation: A review, *Bioelectroch. Bioener.* 41 (1996) 135-160.
- [20] R. Nehmé, C. Atieh, S. Fayad, B. Claude, A. Chartier, M. Tannoury, F. Elleuch, S. Abdelkafi, C. Pichon, P. Morin, Microalgae amino-acid extraction and analysis at nanomolar level using electroporation and CE with laser-induced fluorescence detection, *J. Sep. Sci.* 40 (2017) 558-566.
- [21] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, Accelerated solvent extraction:• a technique for sample preparation, *Anal. Chem.* 68 (1996) 1033–1039.
- [22] J.R.J. Pare, J.M.R. Bélanger, Microwave-assisted process (MAPTM): a new tool for the analytical laboratory, *Trend. Anal. Chem.* 13 (1994) 176-184.
- [23] C. Juin, J.R. Cherouvrier, V. Thiéry, A.-L. Gagez, J.-B. Bérard, N. Joguet, R. Kaas, J.-P. Cadoret, L. Picot, Microwave-assisted extraction of phycobiliproteins from *Porphyridium purpureum*, *App. Biochem. Biotech.* 175 (2015) 1-15.
- [24] M. Mubaraka, A. Shaijaa, T.V. Suchithrab, A review on the extraction of lipid from microalgae for biodiesel production, *Algal Res.* 7 (2015) 117-123.
- [25] K.H. Cha, H.J. Lee, S.Y. Koo, D.-G. Song, D.-U. Lee, C.-H. Pan, Optimization of pressurized liquid extraction of carotenoids and chlorophylls from *chlorella vulgaris*, *J. Agric. Food Chem.* 58 (2010) 793–797.
- [26] M.D. Macías-Sanchez, C. Mantell, M. Rodríguez, E.M.d.l. Ossa, L.M. Lubian, O. Montero, Supercritical fluid extraction of carotenoids and chlorophyll a from *Synechococcus* sp., *J. Supercrit. Fluids* 39 (2007) 323-329.
- [27] N. Grimi, A. Dubois, L. Marchal, S. Jubeau, N.I. Lebovka, E. Vorobiev, Selective extraction from microalgae *Nannochloropsis* sp. using different methods of cell disruption, *Bioresour. Technol.* 153 (2014) 254-259.
- [28] H. Nehmé, R. Nehmé, P. Lafite, E. Duverger, S. Routier, P. Morin, Electrophoretically mediated microanalysis for in-capillary electrical cell lysis and fast enzyme quantification by capillary electrophoresis, *Anal. Bioanal. Chem.* 405 (2013) 9159-9167.
- [29] R. Nehmé, H. Nehmé, G. Roux, D. Cerniauskaite, P. Morin, P. Rollin, A. Tatibouët, Contactless conductivity detection for screening myrosinase substrates by capillary electrophoresis, *Anal. Chim. Acta* 807 (2014) 153-158.
- [30] H. Nehmé, R. Nehmé, P. Lafite, S. Routier, P. Morin, Human protein kinase inhibitor screening by capillary electrophoresis using transverse diffusion of laminar flow profiles for reactant mixing, *J. Chromatogr. A* 1314 (2013) 298-305.
- [31] S. Fayad, R. Nehmé, M. Langmajerová, B. Ayela, C. Colas, B. Maunit, J.-C. Jacquinet, A. Vibert, C. Lopin-Bon, G. Zdeněk, P. Morin, Hyaluronidase reaction kinetics evaluated by capillary electrophoresis with UV and high-resolution mass spectrometry (HRMS) detection, *Anal. Chim. Acta* 951 (2017) 140-150.
- [32] K.P. Vercruysse, A.R. Lauwers, J.M. Demeester, Kinetic investigation of the action of hyaluronidase on hyaluronan using the Morgan-Elson and neocuproine assays, *Biochem.* 310 (1995) 55-59.
- [33] A. Chbani, H. Mawlawi, S. Etahiri, Évaluation de l'activité inhibitrice des extraits d'une algue brune, *Padina pavonica*, récoltée sur les côtes libanaises, *Afr. Sci.* 7 (2011) 91-96.

- [34] F. Omezzine, R. Haouala, A.E. Ayeb, N. Boughanmi, Allelopathic and antifungal potentialities of *Padina pavonica* (L.) extract, *J. Plant Breed. Crop Sci.* 1 (2009) 94-203.
- [35] I. Tuney, B.H. Cadirci, D. Unal, A. Sukatar, Antimicrobial activities of the extracts of marine algae from the coast of Urla (Üzmir, Turkey), *Turk. J. Biol.* 30 (2006) 171-175.
- [36] R.M. Rodriguez-Jasso, S.I. Mussatto, L. Pastrana, C.N. Aguilar, J.A. Teixeira, Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed, *Carbohydr. Polym.* 86 (2011) 1137-1144.
- [37] L. Gourguillon, E. Destandau, A. Lobstein, E. Lesellier, Comparaison de différentes méthodes d'extraction d'acides dicaféoyliques à partir d'une plante halophile, *C. R. Chim.* 19 (2016) 1133-1141.
- [38] L. Jamalzadeh, H. Ghafoori, R. Sariri, H. Rabuti, J. Nasirzade, H. Hasani, M.R. Aghamaali, Cytotoxic effects of some common organic solvents on MCF-7, RAW-264.7 and Human umbilical vein endothelial cells, *Avicenna J. Med. Biochem.* 4 (2016) e33453.
- [39] J. Iqbal, C. Theegalab, Microwave assisted lipid extraction from microalgae using biodiesel as co-solvent, *Algal Res.* 2 (2013) 34-42.
- [40] Y.-M. Dai, K.-T. Chen, C.-C. Chen, Study of the microwave lipid extraction from microalgae for biodiesel production, *J. Chem. Eng.* 250 (2014) 267-273.
- [41] J. Duval, V. Pecher, M. Poujol, E. Lesellier, Research advances for the extraction, analysis and uses of anthraquinones: A review, *Ind. Crops Prod.* 94 (2016) 812-833.
- [42] J. Duval, E. Destandau, V. Pecher, E. Lesellier, Selective enrichment in bioactive compound from *Kniphofia uvaria* by super/subcritical fluid extraction and centrifugal partition chromatography, *J. Chromatogr. A* 1447 (2016) 26-38.
- [43] S. Salmen, J. Hoechstetter, C. Käsbauer, D.H. Paper, G. Bernhardt, A. Buschauer, Sulphated oligosaccharides as inhibitors of hyaluronidases from bovine testis, bee venom and streptococcus agalactiae, *Planta Med.* 71 (2005) 727-732.
- [44] A. Suzuki, H. Toyoda, T. Toida, T. Imanari, Preparation and inhibitory activity on hyaluronidase of fully O-sulfated hyaluro-oligosaccharides, *Glycobiology* 11 (2001) 57-64.
- [45] R.V. Menshova, S.P. Ermakova, S. Rachidi, Amal Al-Hajje, T. N. Zvyagintseva, H.M. Kanaan, Seasonal variations of the composition, structural features, and antitumor properties of polysaccharides from *Padina pavonica* (Lebanon) as a function of composition, *Chem. Nat. Compd.* 47 (2012) 764-769.
- [46] K.S. Girish, K. Kemparaju, S. Nagaraju, B.S. Vishwanath, Hyaluronidase inhibitors: a biological and therapeutic perspective, *Curr. Med. Chem.* 16 (2009) 2261-2288.
- [47] R. Nehmé, P. Morin, Advances in capillary electrophoresis for miniaturizing assays on kinase enzymes for drug discovery, *Electrophoresis* 36 (2015) 2768-2797.
- [48] S. Fayad, R. Nehmé, P. Lafite, P. Morin, Assaying Human Neutrophil Elastase activity by capillary zone electrophoresis combined with laser-induced fluorescence (CZE-LIF), *J. Chromatogr. A* 1419 (2015) 116-124.
- [49] V. Okhonin, E. Wong, S.N. Krylov, Mathematical model for mixing reactants in a capillary microreactor by transverse diffusion of laminar flow profiles, *Anal. Chem.* 80 (2008) 7482-7486.
- [50] S.M. Krylova, V. Okhonin, C.J. Evenhuis, S.N. Krylov, The Inject-Mix-React-Separate-and-Quantitate (IMReSQ) approach to studying reactions in capillaries, *Trend. Anal. Chem.* 28 (2009) 987-1010.
- [51] A.P. Petrov, B.J. Dodgson, L.T. Cherney, S.N. Krylov, Predictive measure of quality of micromixing, *Chem. Commun.* 47 (2011) 7767-7769.

- [52] S. Fayad, R. Nehmé, B. Claude, P. Morin, Human neutrophil elastase inhibition studied by capillary electrophoresis with laser induced fluorescence detection (LIF) and microscale thermophoresis (MST), *J. Chromatogr. A* 1431 (2016) 215–223.
- [53] P. Scodeller, Hyaluronidase and other extracellular matrix degrading enzymes for cancer therapy: New uses and nano-formulations, *J. Carcinog. Mutagen.* 5 (2014) 1-5.

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Fig 1: Electropherograms of the best conditions of extractions of *Padina pavonica* extracts using MAE (a), SFE (b), electroporation extraction (c) and PLE (d). For extraction conditions see section 3 and for pre-capillary and separation conditions see sections 2.4 & 2.5. For each technique a blank assay was conducted without addition of the extract. Peak identification: tetrasaccharide (Tet) and hyaluronic acid (HA).

Fig 2: a) Electropherograms obtained for the inhibition of HA hydrolysis by hyaluronidase with different concentrations of PLE ethyl acetate (I) and PLE water (II) extracts. **b)** Dose-response curves obtained for hyaluronidase inhibition with PLE ethyl acetate extracts using offline assay (I) and PLE water extracts using offline (II.b.1) and online (II.b.2) assays. TDLFP conditions: injection in 7 steps at 0.5 psi: 1. IB (5 s; ~5 nL), 2. BTH (4 s; ~4 nL), 3. water extract (~4 nL), 4. HA (~4 nL), 5. water extract (~4 nL), 6. BTH (~4 nL) and 7. IB (15 s; ~15 nL); t_{wait} : 60 min. Each point represents an average velocity (n=3). Other conditions as **Fig 1**.

Table 1. Inhibition percentage (I %) of hyaluronidase activity obtained by microwave-assisted extraction

Ethyl acetate extract (0.2 mg.mL ⁻¹)			
Temperature \ Time	40°C	60°C	80°C
2 min	86%	75%	65%
5 min	80%	72%	61%
* Water extracts at 60, 100 and 120°C and 2 or 5 min induce 100% hyaluronidase inhibition			

Table 2. Hyaluronidase inhibition by SFE extracts obtained with various extraction solvents

Solvent mixture (v/v)	Extraction time (min)	Inhibition (%)
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CO ₂ -EtOH (95/5)		46
CO ₂ -EtOH (80/20)	30	62
CO₂-EtOH-water (80/16/4)		90
CO ₂ -EtOH (95/5)		
CO ₂ -EtOH (80/20)	15	70
CO₂-EtOH-water (80/16/4)		

Table 3. Inhibition percentage (I %) of hyaluronidase activity obtained by electroporation extracts according to the applied voltage and extraction time

Applied voltage (V)	Extraction time (min)	I %
6	2	0
12	2	0
24	2	24
	2	53
32	5	52
	10	65

Table 4. HPTLC conditions for each chemical family

Family	Elution mixture	Visualization reagent	Standard	Results	
				Ethyl acetate extract	Water extract
Terpenes	9 mL n-hexane / 1 mL ethyl acetate	Anisaldehyde	Ursolic acid	x	x
Polyphenols	9 mL ethyl acetate / 5 mL MeOH / 0.1 mL formic acid	Neu+Peg	Gallic acid	√	x
Flavonoids	9 mL ethyl acetate / 1 mL MeOH / 0.1 mL formic acid	Neu+Peg	Quercetin	x	x
Lipids	2 mL heptane / 2 mL chloroforme / 0.5 mL MeOH	Primuline in acetone/water (80/20)	cholesterol	√	x
Amino acids	7 mL butanol / 1.8 mL acetic acid / 1.2 mL water	Ninhydrin	Alanine	x	x

*√ : Presence of tested compounds ; **x** : Absence of tested compounds

Table 5. Optimum extraction conditions and corresponding hyaluronidase inhibition percentage (I %)

Extraction technique	Optimum extraction conditions	I %
Microwave-assisted extraction (MAE)	Water at 60°C for 2 min	100
Supercritical fluid extraction (SFE)	CO ₂ -EtOH-water (80/16/4) at 30°C for 30 min	90
Electroporation extraction	Water at 32 V for 10 min	65
Pressurized liquid extraction (PLE)	Water at 60°C for 2 min with 2 extraction cycles	100

a. Microwave assisted extraction (MAE)

b. Supercritical fluid extraction (SFE)

c. Electroporation extraction

d. Pressurized liquid extraction (PLE)

Figure 1.

I-PLE ethyl acetate extract

II-PLE water extract

Figure 2.