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

4

5 Syntia Fayad¹, Reine Nehmé^{1*}, Mona Tannoury², Eric Lesellier¹, Chantal Pichon³, Philippe
6 Morin¹

7

8 ¹*Institut de Chimie Organique et Analytique (ICOA), Université d'Orléans - CNRS FR 2708 -*
9 *UMR 7311, Orléans, France*

10 ²*Faculty of Science II, Department of Biology, Lebanese University, 90656, Jdeidet El Metn,*
11 *Fanar, Lebanon*

12 ³*Centre de Biophysique moléculaire, CNRS UPR4301 and Université d'Orléans, France*

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

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27 * Corresponding author: Dr. Reine Nehmé, Institut de Chimie Organique et Analytique
28 (ICOA), Université d'Orléans - CNRS, BP 6759, rue de Chartres, 45067 Orléans cedex 2,
29 France.

30 e-mail: reine.nehme@univ-orleans.fr ; Tel: +33-2-38-49-27-75 ; Fax: +33-2-38-41-72-81

31

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 $0.46 \pm 0.04 \text{ mg}\cdot\text{mL}^{-1}$ and $137.1 \pm 0.3 \text{ nM}\cdot\text{s}^{-1}$ ($r^2=0.99$; $n=3$), 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 $0.04 \pm 0.01 \text{ mg}\cdot\text{mL}^{-1}$ ($r^2=0.99$; $n=3$). This value is comparable to the one obtained for *Eisenia bicyclis* phlorotannin fractions ($IC_{50} = 0.03 \text{ mg}\cdot\text{mL}^{-1}$), which makes *Padina pavonica* bioactivity very promising.

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Highlights:

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64 1. Development of online assay for hyaluronidase inhibition by CE

65 2. Activity of a brown alga, *Padina pavonica*, against hyaluronidase was studied

66 3. Different extraction techniques were used and optimized to obtain active extracts

67 4. IC_{50} of water and ethyl acetate extracts toward hyaluronidase evaluated by CE

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74 **1. Introduction**

75 The extracellular matrix of the skin consists of a complex and dynamic network of
76 macromolecules composed of a variety of proteins such as collagen and elastin as well as
77 proteoglycans to which glycosaminoglycan (GAG) chains are attached. The most common
78 GAG in the dermis is hyaluronic acid (HA) or hyaluronan [1,2]. Since it is extremely
79 hydrophilic, HA has a high hydration capacity contributing to the viscoelastic properties of
80 the skin. Hyaluronidase is the endoglycosidase enzyme that randomly cleaves internal β -N-
81 acetyl-hexosamine and glucosidic linkages in hyaluronic acid (HA) [3,4]. In addition to its
82 important role in skin aging, hyaluronidase has been successfully utilized in ophthalmic
83 surgery and is now being implemented in dermatosurgery and other surgical disciplines [5].
84 Finding new effectors of this enzyme is of great interest. Shibata et al. [6] evaluated the effect
85 of *Eisenia bicyclis* brown alga phlorotannins on hyaluronidase by using an *in vitro* assay. The
86 8,8'-bieckol, an eckol-type phlorotannin, was shown to have the strongest inhibitory effect
87 with an IC_{50} of $0.03 \text{ mg}\cdot\text{mL}^{-1}$. Valentao et al. [7] also found that the phlorotannin extracts
88 from four brown seaweeds, particularly from *Fucus spiralis*, had anti-hyaluronidase activity
89 with an IC_{50} equal to $0.73 \text{ mg}\cdot\text{mL}^{-1}$. Moreover, Ratnasooriya et al. [8] showed that Sri Lankan
90 black tea (*Camellia sinensis* L.) brew had a moderate anti-hyaluronidase activity ($IC_{50} = 1.09$
91 $\pm 0.12 \text{ mg}\cdot\text{mL}^{-1}$).

92 Algae are rich in bioactive compounds which increase their importance nowadays in many
93 fields [9-11]. In this work, *Padina pavonica* alga was studied for its inhibition activity
94 towards hyaluronidase. This brown alga is commonly known as Peacocks tail and is found in
95 the Atlantic Ocean and in the Mediterranean Sea. *Padina pavonica* alga is known for its
96 capacity of restoring the calcium metabolism in the epidermis and maintaining a youthful
97 aspect of the skin by promoting the renewal of GAGs, thereby boosting their protective action
98 [12]. Moreover, Ktari and Guyot [13] evaluated the cytotoxic activity of *Padina pavonica*
99 dichloromethane extracts against human buccal epidermal carcinoma cells and found that 0.01
100 $\text{mg}\cdot\text{mL}^{-1}$ dose was sufficient to obtain 100% inhibition of tumor cell survival. The antioxidant
101 and antifungal activities of this alga were studied by Chbani et al. [14] after maceration and
102 soxhlet extractions with methanol. Ethyl acetate fraction was adequately prepared and showed
103 the best antifungal activity toward *Candida glabrata* and *Candida krusei* species. This activity
104 was referred to phenolic compounds. Mohamed et al. [15] studied the antiviral activity of
105 *Padina pavonica* against *herpes simplex virus* (HSV) and *hepatitis A virus* (HAV). The water

106 extract of sulfated heteropolysaccharide fraction showed an important antiviral activity (72-
107 73%) at 0.02 mg.mL⁻¹.

108 Nowadays, different extraction techniques can be used. The conventional one such as soxhlet
109 extraction is very time consuming (several hours) and requires relatively large quantities of
110 solvent which cause environmental problems and high operating costs. Recently, several
111 novel extraction techniques such as pressurized liquid extraction (PLE), microwave-assisted
112 extraction (MAE), supercritical fluid extraction (SFE) as well as ultrasonic- and
113 electroporation - assisted extractions have been developed [16-20]. PLE is an automated
114 extraction technique developed by Richter et al. [21] in which elevated temperatures and
115 pressures are applied to achieve extractions in short periods of time. MAE utilizes microwave
116 energy for heating, and thus increasing the mass transfer rate of the solutes from the sample
117 matrix into the solvent. The first extraction of a natural product by MAE was achieved by
118 Pare in 1994 [22]. These techniques are widely used to extract biologically active molecules
119 [23-25]. SFE is a promising green technology that can potentially displace the use of
120 traditional organic solvents by carbon dioxide (CO₂) for extraction. CO₂ facilitates a safe
121 extraction due to its low toxicity, low flammability and lack of reactivity [18,26]. SFE
122 presents additional advantages such as operating at a low temperature that reduces the thermal
123 degradation of labile compounds and high selectivity in the extraction of target compounds.
124 Recently, a lab-made apparatus using electroporation-assisted extraction was developed in our
125 group to extract amino acids from microalgae cells by applying a continuous voltage of few
126 volts [20]. Electroporation is an efficient process for breaking cell membranes that was also
127 used for extracting proteins and small molecules from bacterial and eukaryote cells [27,28].

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

139

140 **2. Materials and methods**141 **2.1. Chemicals**

142 All reagents were of analytical grade and used as received without further purification.
143 Ammonium acetate ($C_2H_3O_2NH_4$, purity $\geq 98\%$), hyaluronidase type I-S from bovine testes
144 (BTH, 400-1000 units.mg⁻¹ solid), sodium acetate (CH_3COONa , purity $\geq 99\%$), sodium
145 hydroxide (NaOH, purity $\geq 98\%$) and oligohyaluronic acid 4 (oligo-HA4 or tetrasaccharide
146 (**Tet**), $C_{28}H_{44}N_2O_{23}$) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).
147 Hyaluronic acid sodium salt (HA) from *streptococcus pyrogenes* was purchased from Merck
148 Millipore (Molsheim, France). Glacial acetic acid (CH_3CO_2H), ammonia (NH_3 , purity 28%),
149 diethyl ether (Et_2O), ethanol (EtOH), ethyl acetate ($C_4H_8O_2$) and sulfuric acid (H_2SO_4 , purity
150 96%) were purchased from VWR International (Fontenay-sous-Bois, France). Petroleum ether
151 was purchased from Carlo Erba (Val-de-Reuil, France). The CO_2 was supplied by Linde
152 (Munich, Germany). Ultra-pure water (18 M Ω .cm) was produced from an Elga apparatus
153 (Elga, Villeurbanne, France). Syringes and hydrophilic polyvinylidenedifluoride (PVDF)
154 Millex-HV Syringe Filters, pore size 0.45 μm , were purchased from Millipore (Molsheim,
155 France).

156 **2.2. Solutions**

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

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

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

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

172 **2.3. Plant materials**

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

180 **2.4. Capillary electrophoresis conditions**

181 The capillary electrophoresis system used was a PA800+ automated CE instrument from AB
182 Sciex (Brea, CA, USA) equipped with a photodiode array detection system. The control of CE
183 was performed using 32 Karat software (Beckman Coulter). All through this study, uncoated
184 fused-silica capillaries (60 cm x 49.8 cm x 50 µm) purchased from Polymicro Technologies
185 (Phoenix, AZ, USA) were used. New capillaries were initially conditioned by performing the
186 following rinse cycles: 1 M NaOH (20 min), water (5 min) and BGE (10 min). Between two
187 runs, the capillary was flushed with NaOH 1 M (1 min), water (0.5 min), and BGE (3 min). At
188 the end of each working day, the capillary was rinsed with NaOH 1 M (10 min) and water (10
189 min) to ensure a good cleaning of the inner capillary surface, and stored overnight in water.
190 All rinse cycles were carried out at 30 psi. Hydrodynamic injections were performed at the
191 anodic side of the capillary and normal polarity mode was used with a separation voltage of +
192 20 kV. Electrophoretic analyses were realized at 25°C in an ammonium acetate buffer (pH
193 9.0, ionic strength of 50 mM). The detection of the substrate (HA) and the products was
194 performed at 200 nm (bandwidth 10 nm). The corrected-peak area (CPA) of an analyte
195 represents the peak area, which has been corrected for migration time. The relative standard
196 deviation (RSD) of the corrected peak-areas and of the migration times was used to assess
197 intra-day and inter-day repeatabilities. The limit of quantification (LOQ) was calculated as the
198 concentration corresponding to a signal-to-noise ratio of 10.

199 **2.5. Offline (pre-capillary) enzymatic assays**

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

205 μL) of HA ($0.4 \text{ mg}\cdot\text{mL}^{-1}$, corresponding to the K_m value in the literature of $0.46 \text{ mg}\cdot\text{mL}^{-1}$
 206 [32]) was added to the mixture and the incubation time was 1 hour. The reaction was then
 207 stopped by heating during 10 min at 90°C . After cooling, 5 nL (0.5 psi for 5 sec) of the
 208 reaction mixture was introduced into the capillary. Blank assays, without the addition of the
 209 enzyme, the substrate and/or the extract were conducted before each set of CE analysis.

210 **2.6. Online enzymatic assays**

211 Transverse diffusion of laminar flow profiles (TDLFP) approach was used for in-capillary
 212 mixing of hyaluronidase with hyaluronic acid. After filling the capillary with the BGE, the
 213 reagents were introduced at the inlet side of the capillary as follows: IB (0.5 psi for 5 s; ~ 5
 214 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
 215 nL). As for the offline assays, the incubation time was 1 h and a voltage of +20 kV was
 216 applied for separation. The order of the plugs was based on the diffusion coefficient (D_m) of
 217 each reagent. These coefficients were estimated using the Stokes-Einstein equation [32]:

$$218 \quad D_m = \frac{K_B \cdot xT}{\eta} \sqrt[3]{\frac{dN_A}{162\pi^2 M}} \quad (1)$$

219 where D_m is the diffusion coefficient ($\text{cm}^2\cdot\text{s}^{-1}$); K_B is the Boltzman constant ($\text{m}^2\cdot\text{g}\cdot\text{s}^{-2}\cdot\text{K}^{-1}$); T is
 220 the temperature (Kelvin); η is the viscosity ($\text{m}^2\cdot\text{s}^{-1}$); d is the density ($\text{kg}\cdot\text{cm}^{-3}$); N_A is the
 221 Avogadro constant (mol^{-1}) and M is the molecular weight of each reactant ($\text{kg}\cdot\text{mol}^{-1}$).

222 According to equation (1), the following results were obtained: D_m (BTH) = $1.4 \times 10^{-6} \text{ cm}^2\cdot\text{s}^{-1}$
 223 and D_m (HA) = $3.4 \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$.

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

228 **2.7. Kinetic analysis of hyaluronidase reaction - determination of V_{max} , K_m and IC_{50}**

229 For online assays, BTH was incubated with a range of concentrations of HA including the K_m
 230 value reported in the literature ($K_m = 0.46 \text{ mg}\cdot\text{mL}^{-1}$ [32]). Thus, the concentrations of HA
 231 ranged from 0.10 to $1.2 \text{ mg}\cdot\text{mL}^{-1}$. Each point was repeated three times ($n=3$). The initial
 232 reaction rate V_i ($\text{nM}\cdot\text{s}^{-1}$) was calculated as the ratio of the substrate formed *per* time interval.
 233 The nonlinear curve-fitting program PRISM[®] 5.04 (GraphPad, San Diego, CA, USA) was
 234 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 ($\text{nM}\cdot\text{s}^{-1}$), K_m is the Michaelis-Menten constant ($\text{mg}\cdot\text{mL}^{-1}$), V_{\max} is the maximum reaction velocity ($\text{nM}\cdot\text{s}^{-1}$) and $[S]$ is the substrate concentration ($\text{mg}\cdot\text{mL}^{-1}$).

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 $3\text{ mL}\cdot\text{min}^{-1}$ (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

265 A lab-made device already developed by our group was used for amino acid extraction studies
266 [20]. It consisted of two parallel platinum electrode wires, each one having a surface area of
267 0.6 cm^2 (1 cm length x 0.1 cm radius), connected to the positive and negative outlets of a DC
268 (AL841B 30VA – ELC, Annecy, France). The inter-electrode gap was 1.7-1.8 cm. 50 mg of
269 *Padina pavonica* alga was suspended in 20 mL of distilled water (2.5 g.L^{-1}) and placed in a
270 glass vessel, on a magnetic stirrer. The sample was stirred constantly to prevent cells from
271 settling out of suspension over the time-course of extraction and to maintain steady the
272 diffusion around the electrodes. Extraction was performed by applying a voltage (6, 12, 24
273 and 32 V) to the suspension at different duration times [20] (Table 3).

274 **2.11. PLE**

275 PLE was performed with an ASE 100 system from Dionex (Voisins le Bretonneux, France)
276 equipped with 5 mL stainless steel cells. 650 mg of alga were mixed homogeneously with the
277 same weight of diatomaceous earth and placed into the extraction cell. The extraction cells
278 were placed into the PLE system and the extraction conditions were selected as follows: a
279 static time of 10 min, followed by a flush elution with 65% volume and a nitrogen purge of
280 60 s with 2 cycles of extraction. The pressure was fixed at 150 bars. Different parameters
281 were optimized to obtain the most efficient anti-hyaluronidase extract. Then, ethyl acetate,
282 ethanol, petroleum ether, and water were tested as extraction solvents at 60°C . Extraction
283 temperature and cycle number effect were evaluated for the optimal extraction solvent.

284 **2.12. HPTLC analysis**

285 HPTLC analyses were done on $10 \times 20 \text{ cm}$ HPTLC plates (Merck, Germany) by a CAMAG
286 (Muttens, Switzerland) ATS4 Automatic TLC Sampler controlled by Win-CATS software
287 and were observed with a Reprostar 3 illumination unit. 10 μL of extract solutions and 4 μL of
288 standard solutions each at 1 mg.mL^{-1} were laid down. Plate elution was performed in a
289 horizontal developing chamber. After development, the plate was dried and observed under
290 254 and 366 nm light. The plate was then sprayed with different specific or non-specific
291 reagents. For each compound family, elution mixture, visualization reagent and standards are
292 mentioned in Table 4.

293

294 **3. Results and discussion**

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

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

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

309 **3.1. MAE- optimization**

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

322 **3.2. SFE optimization**

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

331 to bring substantial changes on the solvation properties of supercritical CO₂. Two extraction
332 durations, 15 and 30 min, were tested. Hyaluronidase inhibition results are reported in Table
333 2. It can be seen that in the presence of water as CO₂ modifier, even at only 4%,
334 hyaluronidase was almost completely inhibited by 0.2 mg.mL⁻¹ of extract; I = 90% (Tables 2
335 & 5 and Fig.1.b). These results confirm those obtained in the previous section using MAE
336 technique.

337 **3.3. Electroporation extraction optimization**

338 Electroporation has been recently developed by our group [20] as a simple, non-expensive
339 and efficient extraction procedure for *Dunaliella Salina* microalga. The efficiency of this
340 technique depends on the applied voltage during a certain time. Water is used as the
341 extraction solvent which is, therefore, suitable for the extraction of polar molecules and for
342 cosmetic applications. Different direct voltages were first applied (6, 12, 24 and 32 V) with 2
343 min of extraction time as mentioned in Table 3. In the first assays, NaCl was added to the
344 suspension mixture to maintain a constant current between the different extraction
345 experiments. Hyaluronidase was activated by the obtained extracts, which was attributed to
346 the presence of NaCl reported elsewhere as an activator of this enzyme [3]. In the absence of
347 additional NaCl, CE inhibition results showed that a voltage of 32 V applied during 2 min was
348 the optimum for extracting molecules responsible for anti-hyaluronidase activity; I = 53% at
349 0.2 mg.mL⁻¹ (Tables 3 & 5). At this voltage, increasing the extraction time to 5 and 10 min
350 allowed a relatively small improvement of the inhibition to 65% (Fig 1.c). Water extracts
351 obtained by electroporation are therefore less efficient than those obtained by MAE and SFE.

352 **3.4. PLE optimization and extract analysis by HPTLC**

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

364 To our knowledge, no work emphasizing the anti-hyaluronidase effect of *Padina pavonica* is
365 described in the literature. Ethyl acetate and water PLE extracts were analyzed by HPTLC to
366 obtain information about their chemical content (Table 4). Several families of natural
367 compounds (terpenes, flavonoids and amino acids) were absent in ethyl acetate and water
368 extracts. On the other hand, polyphenols and lipids were only revealed in ethyl acetate
369 extracts. Indeed, several studies mentioned [14,15] that this solvent mainly extracts phenolic
370 compounds from *Padina pavonica* brown alga whereas water mainly extracts sulfated
371 polysaccharides. The latter compounds are known for their anti-hyaluronidase activity
372 [31,43,44] as well as for their antiviral and antioxidant properties [15,45]. Girish et al. [46]
373 listed the different chemical classes of hyaluronidase inhibitors. Alkaloids,
374 glycosaminoglycans, glycosides as well as oligo- and poly- saccharides are the main polar
375 inhibitors. Further chemical characterization of the active extracts is needed to identify the
376 family or even the molecule(s) responsible for hyaluronidase inhibition.

377 **3.5. Hyaluronidase online kinetic study**

378 CE has become a frequently used tool for miniaturizing enzyme assays using online mode
379 mainly due to its well-recognized low sample consumption [47]. In this study, an online CE
380 assay for monitoring hyaluronidase reaction was developed based on TDLFP for reactant in-
381 capillary mixing (see experimental section). Hyaluronidase, having a lower diffusion
382 coefficient ($D_{m(BTH)} = 1.4 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$) was injected first, followed by hyaluronic acid (D_{m}
383 $_{(HA)} = 3.4 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$). To improve the mixing of the enzyme with the substrate, BTH was
384 injected between two plugs of HA which corresponds to the so-called sandwich mode [48]. A
385 plug of IB was injected at the beginning of the injection sequence to prevent inactivating the
386 hyaluronidase by the BGE (partial filling mode) [49]. This IB plug must have a rectangular
387 shape to avoid its transversal diffusion, so its injection must occur for a relatively long time (5
388 s). A plug of IB was also injected (0.5 psi for 15 s) at the end of the injection sequence to
389 improve overlapping of reactant plugs by increasing the length of longitudinal plug interfaces
390 [50]. In this sequence, two plugs of BTH, one plug of HA and one plug of IB were diffusing
391 axially through the parabolic injection zones occupying then a final volume that is certainly
392 higher than their initial injection zone [51]. BTH and HA concentrations are thus expected to
393 be diluted inside the capillary by a factor of 2/3 and 3, respectively.

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

397 ($r^2 = 0.94$; $n=3$), respectively. The values were comparable to those reported in literature [32]
398 using a spectrophotometric assay ($V_{\max} = 126 \text{ nM}\cdot\text{s}^{-1}$ and $K_m = 0.46 \text{ mg}\cdot\text{mL}^{-1}$) and to those
399 obtained by the offline CE-UV/HRMS assay ($V_{\max} = 155.7 \pm 0.2 \text{ nM}\cdot\text{s}^{-1}$ and $K_m = 0.49 \pm 0.02$
400 $\text{mg}\cdot\text{mL}^{-1}$; $n=3$), which validates the developed online assay.

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

412

413 4. Conclusion

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

427

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589
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592 **List of figures**

593

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

599 **Fig 2: a)** Electropherograms obtained for the inhibition of HA hydrolysis by hyaluronidase
 600 with different concentrations of PLE ethyl acetate (I) and PLE water (II) extracts. **b)** Dose-
 601 response curves obtained for hyaluronidase inhibition with PLE ethyl acetate extracts using
 602 offline assay (I) and PLE water extracts using offline (II.b.1) and online (II.b.2) assays.

603 TDLFP conditions: injection in 7 steps at 0.5 psi: 1. IB (5 s; ~5 nL), 2. BTH (4 s; ~4 nL), 3.
 604 water extract (~4 nL), 4. HA (~4 nL), 5. water extract (~4 nL), 6. BTH (~4 nL) and 7. IB (15
 605 s; ~15 nL); t_{wait} : 60 min. Each point represents an average velocity (n=3).

606 Other conditions as **Fig 1**.

607

608

609

610 **Table 1.** Inhibition percentage (I %) of hyaluronidase activity obtained by microwave-
 611 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			

612

613

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

615

Solvent mixture (v/v)	Extraction time (min)	Inhibition (%)
-----------------------	--------------------------	----------------

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)		

616

617

618

619 **Table 3.** Inhibition percentage (I %) of hyaluronidase activity obtained by electroporation

620 extracts according to the applied voltage and extraction time

621

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

622

623

624

Table 4. HPTLC conditions for each chemical family

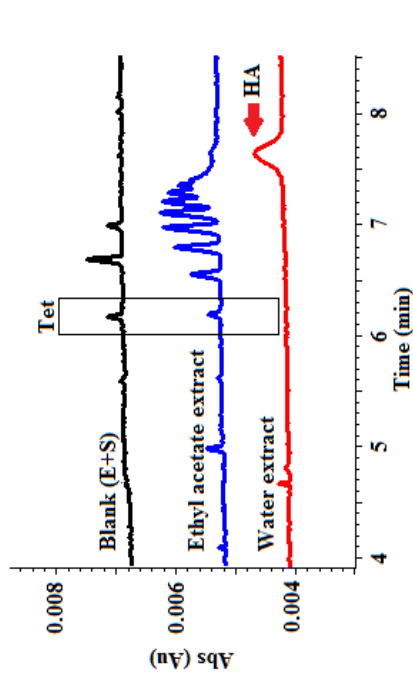
Family	Elution mixture	Visualization reagent	Standard	Results	
				Ethyl acetate extract	Water extract
Terpenes	9 mL n-hexane / 1 mL ethyl acetate	Anisaldehyde	Ursolic acid	x	x
Polyphenols	9 mL ethyl acetate / 5 mL MeOH / 0.1 mL formic acid	Neu+Peg	Gallic acid	√	x
Flavonoids	9 mL ethyl acetate / 1 mL 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

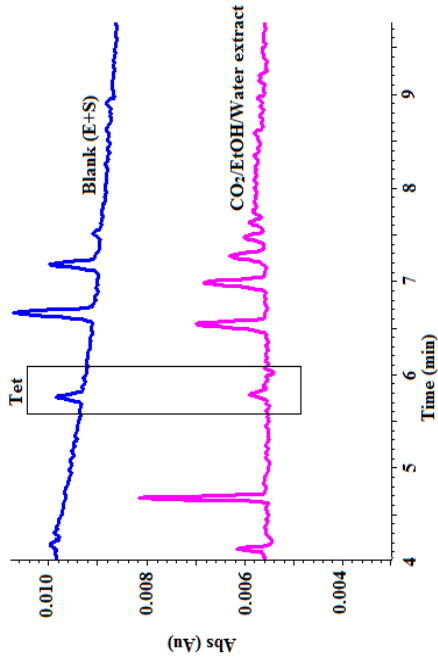
Table 5. Optimum extraction conditions and corresponding hyaluronidase inhibition percentage (I %)

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

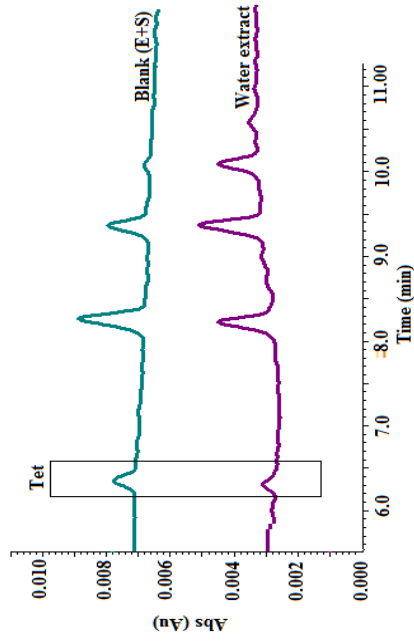
a. Microwave assisted extraction (MAE)



b. Supercritical fluid extraction (SFE)



c. Electroporation extraction



d. Pressurized liquid extraction (PLE)

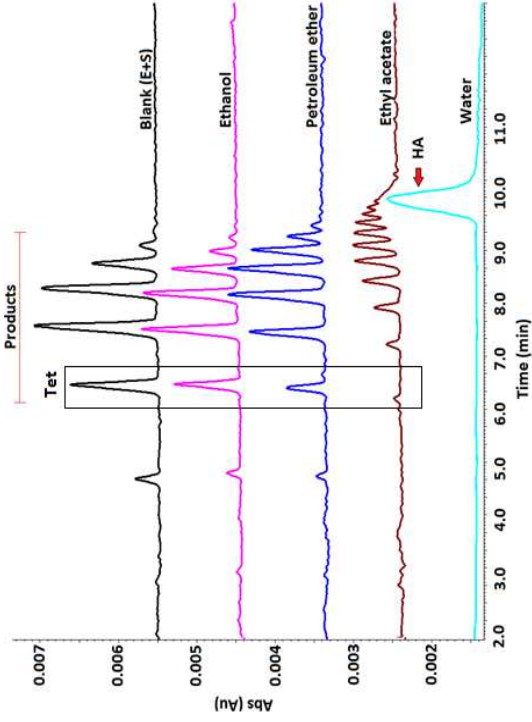
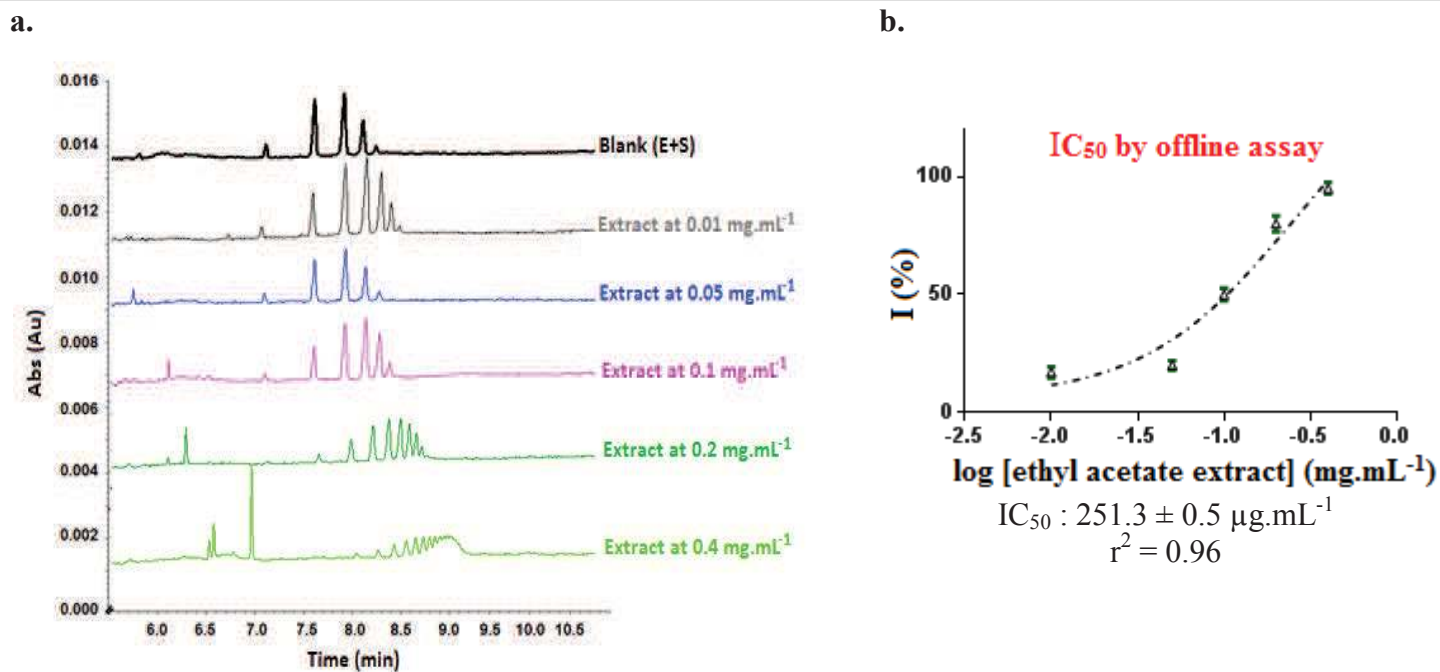


Figure 1.

I-PLE ethyl acetate extract



II-PLE water extract

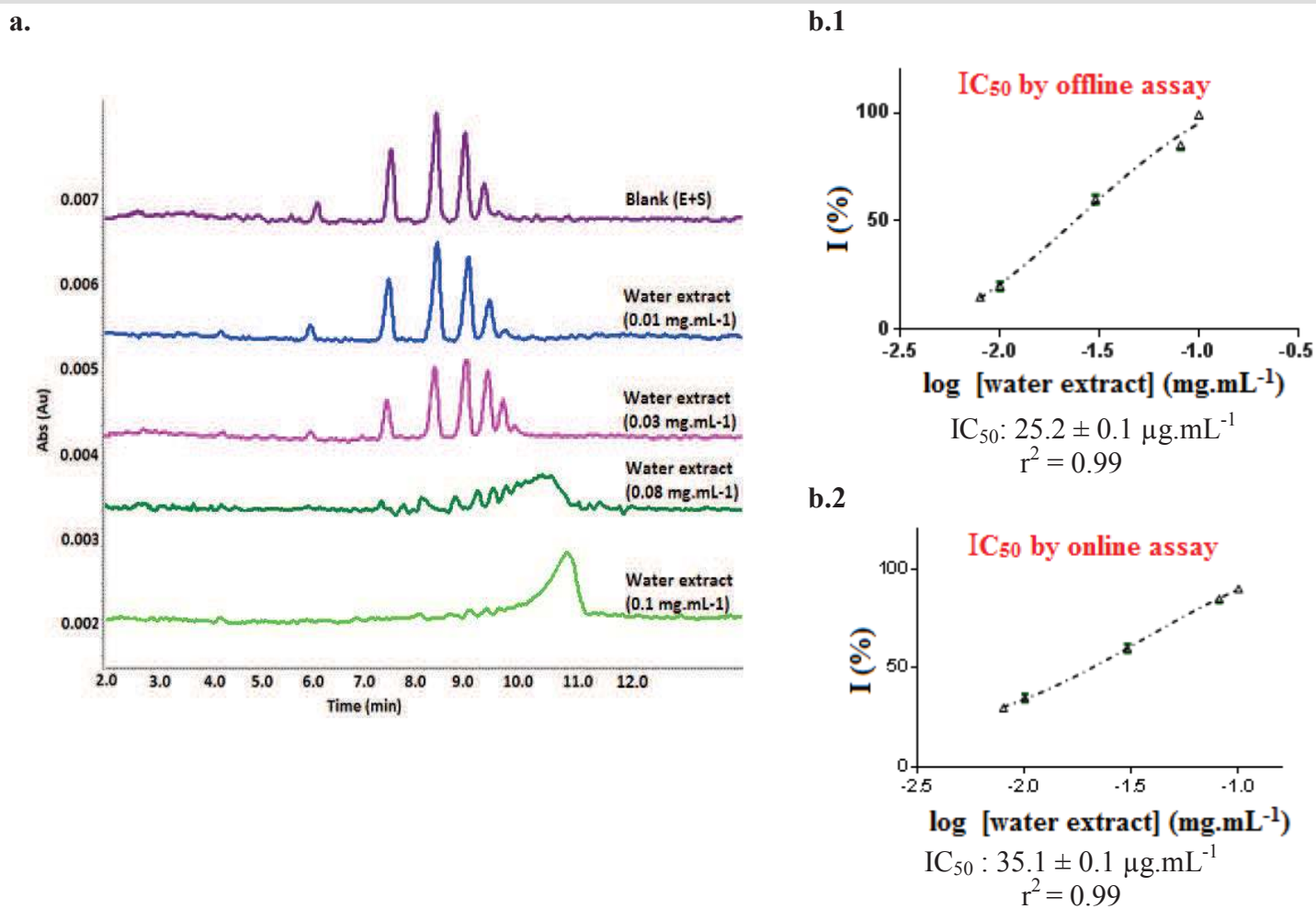


Figure 2.