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1 **Antibacterial activity of isolated phenolic compounds from cranberry (*Vaccinium*
2 *macrocarpon*) against *Escherichia coli***

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26 **Abstract**

27 Phenolic compounds from a cranberry extract were isolated in order to assess their contribution
28 to the antibacterial activity against urophatogenic strains of *Escherichia coli* (UPEC). With this
29 purpose, a total of 25 fractions from a cranberry extract were isolated using semipreparative
30 high performance liquid chromatography (HPLC) and characterized based on the results
31 obtained by reversed-phase HPLC coupled to mass spectrometry detection. Then, the effect on
32 UPEC surface hydrophobicity and biofilm formation of the cranberry extract as well as the
33 purest fractions (a total of 13) was tested. As expected, the whole extract presented a powerful
34 antibacterial activity against UPEC while the selected fractions presented different behavior.
35 Myricetin and quercitrin significantly decreased ($p < 0.05$) *E. coli* biofilm formation compared
36 with the control, while dihydroferulic acid glucuronide, procyanidin A dimer, quercetin
37 glucoside, myricetin and prodelphinidin B led to a significant decrease on the surface
38 hydrophobicity compared with the control. The results suggest that apart from
39 proanthocyanidins, other compounds, mainly flavonoids, can act against *E. coli* biofilm
40 formation and also modify UPEC surface hydrophobicity *in vitro*, one of the first steps of
41 adhesion.

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44 **Keywords:** cranberry, semipreparative-HPLC, phenolic compounds, adherence, biofilm,
45 surface hydrophobicity, *Escherichia coli*.

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51 **INTRODUCTION**

52 Cranberries (*Vaccinium macrocarpon*) are popularly consumed as part of the human
53 diet both fresh and processed forms. Additionally, their derived extracts are also used, mainly as
54 part of some botanical dietary supplements forms due to their renowned human health benefits[1].
55 Cranberry has proved to be an excellent source of bioactive compounds such as flavonoids
56 (procyanidins, flavonols), and phenolic acids derivatives[2]. Thanks to these health-promoting
57 compounds, cranberry and cranberry-based products consumption has been correlated with
58 recurrent urinary tract infections (UTIs) prophylaxis[3,4]. UTI has been defined as the presence of
59 significant number of pathogenic bacteria or organisms in the urinary system and it is
60 considered the most common type of infection in the body, which affects women in a greater
61 extent than men[5]. *Escherichia coli* (*E. coli*) is the main responsible bacterial species for the
62 appearance of this infection, and causes more than 80 percent of all acquired UTIs in the
63 community[6]. Concretely, the ability of urophatogenic *Escherichia coli* (UPEC) to form biofilm
64 has been strongly associated with recurrent UTIs[7, 8] and there have been proven that surface
65 hydrophobicity is conductive to adhesion to surfaces and to penetration of host tissues[9] since
66 bacteria have developed many different ways to use hydrophobic effect in order to adhere to
67 substrata, such as previously described by Doyle *et al*[10]. The importance of biofilms in public
68 health is related to the decreased susceptibility to antimicrobial agents that biofilm-associated
69 microorganisms exhibit. This is the case of *E. coli* which has shown to be increasingly resistant
70 to some of the antibiotics currently used in the treatment of UTIs[11,12]. In addition, the public
71 interest in herbal medicines and natural products is still growing. For this reason, researchers
72 have concluded the re-evaluation of first and second-line therapies for the treatment of UTIs
73 becomes to be pivotal[13]. Consequently, the antimicrobial effect of cranberry products and their
74 phenolic compounds have been widely studied, especially to develop new healthy food
75 ingredients, functional foods, nutraceuticals, and pharmaceuticals[14]. The most accepted theory
76 about the mechanism of action of cranberry compounds for the promotion of urinary tract health

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77 is based on the effects of fructose and PACs in inhibiting the adherence of type 1 and P fimbriae
78 of *E. coli* to the uroepithelial cell receptors[15,16]. Without adhesion, the bacteria cannot infect the
79 mucosal surface. Despite a large number of studies highlighted that there are synergisms
80 between different compounds present in cranberry extracts[17-19], other authors such as Hisano *et*
81 *al.* concluded that the use of the whole cranberry for UTIs prevention was not scientifically
82 supported, and for that reason, it is pointed out the necessity of research focused on bioactive
83 compounds from cranberry instead of the entire fruit[3]. However, the isolation of simultaneous
84 compounds from cranberry extracts is an arduous task due to its complexity. Reversed-phase
85 semipreparative high performance liquid chromatography (semipreparative-HPLC) has been
86 increasingly used once possesses an interesting target separation ability, great efficiency and
87 high recovery[20], and therefore can be a valuable tool to solve the aforementioned difficulty.

88 In this sense, the aims of the present research were to fractionate phenolic compounds
89 from a cranberry extract by semipreparative-HPLC and to give new insights into their
90 contribution to the antibacterial effect by testing the *in vitro* effect of the entire extract and the
91 isolated fractions against *E. coli* surface hydrophobicity and biofilm formation.

92 **RESULTS AND DISCUSSION**

93 **Isolation of phenolic compounds from cranberry extracts by semipreparative-**
94 **HPLC and characterization of fractions by HPLC-ESI-MS.**

95 Natural extracts usually consist of hundreds of compounds, and the isolation of
96 particular components presents unique problems because the methods used to isolate them are
97 based mainly on their polarity. The similarity of some polyphenolic structures makes that
98 compounds elute at similar retention times, making difficult their separation. For that reason,
99 only few studies have focused on the chromatographic methods for the isolation of multiple
100 compounds simultaneously. In this regard, semipreparative-HPLC is a robust, versatile, and
101 usually rapid technique by which compounds can be purified from complex mixtures[21].

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102 In the current research, the analytical HPLC method previously developed for the
103 characterization of phenolic compounds from cranberry extracts[19] was scaled-up to
104 semipreparative-HPLC scale. Different gradients were tested to enhance the separation of the
105 compounds (data not shown), selecting as optimum the method described in "experimental"
106 section. Figure 1 shows the UV chromatogram of the cranberry extract under study acquired
107 with the proposed method, where the fractions collected are indicated according to their elution
108 order.

109 The isolated fractions were subsequently analyzed by HPLC-ESI-QTOF-MS in negative
110 ionization mode. Characterization strategy was carried out by generation of the candidate
111 molecular formula with a mass accuracy limit of 5 ppm, considering their MS spectra
112 determined by quadrupole time-of-flight mass spectrometer (QTOF–MS), and also comparing
113 with those of authentic standards whenever available and data from the literature. Databases
114 such as SciFinder Scholar (http://scifinder.cas.org), MassBank (http://massbank.jp), and
115 METLIN Metabolite Database (http://metlin.scripps.edu) were consulted in order to acquire
116 chemical structure information.

117 Despite the scarcity of literature on the fractionation of cranberry using
118 semipreparative-HPLC makes difficult to contrast our optimized method with others, and the
119 results could not be comparable, the optimized method allowed obtaining 25 fractions from the
120 cranberry extract (Table 1), which were composed predominantly by procyanidins (PACs) and
121 flavonols. Even though the difficulty in separating and purifying PACs has been previously
122 highlighted[22], the current method allowed isolating some of them, including A-type procyanidin
123 dimmers, an A-type procyanidin trimer (cinnamtannin B1) and a gallocatechin dimer
124 (prodelphinidin). PACs are the most typical compounds characterized in cranberry, noteworthy
125 for their antioxidant activity, although they may also present other pharmacological and
126 medicinal properties such as anti-carcinogenic, anti-inflammatory, and vasodilator[23]. Isolated
127 cranberry flavonols included quercetin derivatives which have been previously demonstrated to
128 have both *in vivo* and *in vitro* antioxidant, anti-inflammatory, anticancer, and antidiabetic

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129 activities[24]. In addition, four myricetin derivatives were characterized. These compounds are
130 also common dietary flavonoids which have demonstrated antioxidant, cytoprotective, antiviral,
131 antimicrobial, anticancer and antiplatelet activities[25]. Apart from these compounds, one
132 hydroxicinnamic acid derivative (dihydroferulic acid glucuronide) was isolated.

133 Among these 25 eluted fractions, 13 were chosen in order to test their antibacterial
134 activity against *E. coli, namely* F: 6, 8, 9, 11, 13–16, 18, 19, 21, 23, and 25. These fractions
135 were selected on the basis of their purity, due to they showed a purer composition than the rest,
136 presenting up to two target phenolic compounds. HPLC-ESI-QTOF-MS chromatograms from
137 these nearly pure fractions are displayed in Figure 2. Semipreparative-HPLC allowed getting
138 1.1 mg of F6, F8, and F18; 0.9 mg of F9 and F15;1.7 mg of F11; 1.5 mg of F13; 1 mg of F14;
139 0.7 mg of F16 and F21; 0.6 mg of F19 and F25; and 0.5 mg of F23. Different concentrations
140 tested are depicted in Table S2 (supplementary information). The use of different concentrations
141 of each fraction was established in order to simulate their contribution in the whole extract.

142 **Antibacterial activity**

143 Although some authors reported that cranberry does not have any effect against Gram-
144 negative bacteria pathogens such as *E. coli*[26], most of the research converges on the fact that
145 berries, and especially cranberry and cranberry-based products, have both *in vitro* and *in vivo*
146 antibacterial activity[14,16,19,22,27,28]. As aforementioned, the most accepted mechanism of action of
147 cranberry focuses primarily on its ability to prevent bacterial binding to host cell surface
148 membrane[29], one of the initial steps in the infection process. This process is initially mediated
149 by the electrostatic charge (characterized by determining its zeta potential) and consequently
150 surface hydrophobicity of microorganisms followed by other factors such as formation of
151 fimbriae and specific adhesins[30]. Thus, surface physicochemical parameters such as electrostatic
152 charge are then fundamentally important with regard to influencing overall polarity in order to
153 maintain the degree of bacterial surface hydrophobicity necessary for the bacterial adhesion.
154 Subsequently, adhesion of bacteria to host surfaces is finally a key element in the formation of

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155 biofilms that constitutes a protected mode of growth that allows bacteria to survive in hostile
156 environment[31]. For that reason, the effect of the previously isolated fractions as well as the
157 whole extract on biofilm formation and surface hydrophobicity of fourteen UPECs has been
158 tested as a way to evaluate the individual contribution of every compound to the antibacterial
159 activity.

160 Figures 3 and 4 show the mean and standard deviation (SD) of biofilm formation and
161 surface hydrophobicity for *E. coli* after incubation with each isolated fraction and with the
162 cranberry extract, respectively, at two different assayed concentrations. Table S1
163 (supplementary data) summarizes the Wilcoxon matched-pairs signed-ranks analysis for the
164 biofilm formation and surface hydrophobicity of the isolated fractions and the whole extract.

165 After testing the selected fractions, two concentrations of F9 made up of procyanidin
166 type-A dimer, showed a statistically significant increase in biofilm formation compared with the
167 control (Fig. 3). Other research has also described an increase of biofilm formation in four of the
168 20 *E. coli* strains tested after consuming cranberry juice[7] and a reduction of biofilm formation
169 only in one of them. However, F9 did not significantly change surface hydrophobicity. On the
170 other hand, F13 (made up of other isomer of procyanidin type-A dimer) at the highest
171 concentration (dilution A) caused an increase in biofilm formation while both concentrations
172 tested significantly decreased surface hydrophobicity. In any case, it should be pointed out that
173 the hydrophobicity of bacteria can vary even within the same strain depending on the mode and
174 stage of growth[32]. Despite the study of PACs in *E. coli* has been widely described, controversial
175 results are still reported in literature. Foo *et al*. also found a weak activity of procyanidin A2
176 against the inhibition of adherence of *E. coli*[33]. In another study, PACs as a group of compounds
177 inhibited the growth of *E. coli* CM 871, with no inhibition of *E. coli* 50[17]. Foo *et al* also proved
178 the anti-adherent effect of procyanidin trimers[33]. However, no statistical differences were found
179 between F14 (made up of cinnamtannin B1 and quercetin arabinoside) and the control in both
180 assays tested. Prodelphinidin B (F23) also influenced the antibacterial effect against *E. coli* by
181 decreasing the bacteria surface hydrophobicity. Prodephinidins with pyrogallol groups, which

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182 have similar structures to procyanidins except for their hydroxyphenyl group, have reported to
183 have stronger antibacterial activity than procyanidins with the catechol groups[34]. However, the
184 different results obtained from different isolated PACs, reinforce the theory proposed by
185 Schmidt *et al.* who concluded that it was likely that a mixture of several high molecular weight
186 PACs were responsible for the anti-proliferation and anti-adhesion activity.

187 Regarding isolated flavonols, fraction formed by myricetin and quercitrin (F21) was the
188 most active fraction against the *E. coli* biofilm formation and also influenced the decrease in *E.*
189 *coli* surface hydrophobicity. Bacterial hydrophobicity has been proved to be largely influenced
190 by the residues and structures on the surface of the cell[32]. In this way, recent research has
191 pointed out that phytochemicals such as flavonoids can modify bacterial membrane surface
192 hydrophobicity[35] probably based on their ability to complex with extracellular and soluble
193 proteins as well as with bacterial cell walls. Concretely, three mechanisms of action of
194 flavonoids have been proposed: inhibition of nucleic acid synthesis, cytoplasmic membrane
195 damage and inhibition of energy metabolism[36]. Although the anti-adherent effect of myricetin
196 remains controversial, some authors have found that 0.5 mg mL^{-1} of myricetin strongly inhibited
197 the growth of *E. coli*[17]. Only few studies have been carried out in order to assess the flavonoids
198 structure-antibacterial activity relationship. In this sense, some authors concluded that the
199 hydroxylation at position 5 on the A ring and at position 3 on the C ring improves the
200 antibacterial activity of flavones decreasing membrane fluidity[37,38]. These previous results could
201 explain the antibacterial effects that the combination of quercitrin and myricetin (F21) showed
202 in both assays. Cowan *et al.* reported that more lipophilic flavonoids may disrupt microbial
203 membranes[26]. Furthermore, Wojnicz, *et al*. affirmed that flavonoids such as quercetin, reduced
204 biofilm synthesis because they can suppress autoinducer-2 activity, which is responsible for
205 cell-to-cell communication[39]. In particular other authors have described the existence of
206 antibacterial activity of quercetin against *E. coli*[6]. Contrary to these previous findings, F25,
207 formed by pure quercetin, a molecule that has a lipophilic character despite the presence of five
208 hydroxyl groups in its structure, not only did not show statistical differences in UPEC biofilm

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209 formation at two tested concentrations, but also significantly increased the UPEC surface
210 hydrophobicity compared with control at the highest concentration tested (dilution A). Some
211 authors affirmed, in base of their results, that the degree of hydroxylation might affect the
212 antimicrobial activity of phenolic compounds, indicating that the more polar flavonoids, the
213 more antibacterial effect[17]. In the current study, this theory could be applicable when comparing
214 F25 (quercetin) and F21 (quercitrin and myricetin). The addition of one more hydroxyl group on
215 the aromatic ring of myricetin compared with quercetin may be responsible for its antimicrobial
216 activity. Other research attributes its antimicrobial mechanism against Gram-negative to a
217 reaction with DNA or inhibition of protein synthesis bacteria[40,41]. An early theory based on that
218 hydrophobic effect may be the primary driving force for the adhesion of most pathogens was
219 also proposed[42]. However, taking into account the abovementioned case of quercetin, no relation
220 was observed between *E. coli* surface hydrophobicity and biofilm formation rates.

221 Despite the great general interest in glycosylated flavonoids due to their diverse
222 bioactivity, research focused on their antibacterial properties is still at the developmental stage.
223 None of the tested concentrations of F6 (myricetin glucoside) showed any activity against
224 biofilm formation nor modifying surface hydrophobicity. Some authors have pointed out that
225 the glycosylation of flavonoids leads to a loss of activity against some Gram-negative bacteria[43].
226 In addition, early studies concluded that quercetin monosaccharide derivatives showed weak
227 activity against *E. col* [44]. Following with these compounds, other plant extracts such as white
228 garlic extract, which contains a high concentration of quercetin-4-O-glucoside and quercetin-
229 3,4-O-diglucoside, had a large inhibiting activity on the growth of *E. coli*, among other Gram-
230 negative bacteria[6]. The current results show that quercetin derivatives do not always produce the
231 same antibacterial effect. On one hand, fractions 18 and 19, made up of quercitrin isomer and
232 quercitrin (quercetin-3- rhamnoside) respectively, showed different antibacterial activity. While
233 incubation with F18 caused a statistically significant increment of UPEC biofilm formation
234 compared with the control and did not present significant differences on surface hydrophobicity,
235 F19 (quercitrin) did not show statistical differences in biofilm formation rates but produced a

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236 significant reduction on surface hydrophobicity. Taking into account that F19 was tested at
237 lower concentrations than F18, as depicted in table S2 (supplementary information), this fact
238 suggests that the position of sugar moieties influences the antibacterial activity of flavonoids.
239 Previous studies reported that among quercetin glycosides tested, quercetin-3-rhamnoside
240 exhibited the strongest antibacterial activity against Gram-negative bacteria whereas other
241 quercetin glycosides showed weak or no activity against the same Gram-negative bacteria[45]. On
242 the other hand, F15 and F16, made up of quercetin arabinoside isomers, showed similar trends
243 in significant surface hydrophobicity reduction even testing different concentrations (Table S1,
244 supplementary information) while only F16 at 300 μg mL^{-1} (dilution A) significantly increased
245 the biofilm formation rate.

246 In addition, both tested concentrations of fraction F8, made up of mainly dihydroferulic
247 acid glucuronide, also showed a reduction in the hydrophobicity of *E. coli*. In this regard,
248 Borges *et al*. found that ferulic acid had antimicrobial activity against *E. coli* by irreversible
249 changes in membrane properties through hydrophobicity changes that caused local rupture or
250 pore formation in the cell membranes causing the loss of essential intracellular constituents[46].
251 Despite Borges *et al.* also concluded in other study that ferulic acid reduced mass of biofilm
252 formed by Gram-negative bacteria[47], dihydroferulic acid glucuronide did not show statistically
253 differences compared with the control.

254 If we look at the whole extract, the data revealed statistical differences with respect to
255 control in both, biofilm formation and surface hydrophobicity, after incubating UPEC strains
256 with the cranberry extract independent of the concentrations tested (Figure 4). This finding
257 suggests that even at low dosage, cranberry extract presents antibacterial activity *in vitro*. As
258 pointed out along the text, the hydrophobic properties of microbial surfaces are conducive to
259 adhesion and, thus, to penetration of host tissues. Taking into account the capacity of UPEC to
260 form biofilms, it could be expected a positive relationship between hydrophobicity and biofilm
261 formation. However, the nonparametric Kendall's rank correlation disclosed that there was no

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262 trend between surface hydrophobicity and adherence (W=0.236; p=0.019) of UPEC tested after
263 the incubation with cranberry extract. These results could be attributed to the different behavior
264 of each strain. In fact, despite most of UPEC strains are *in vitro* positive for biofilm
265 production[48], it has been previously reported that even the same strain can respond very
266 differently to biofilm formation depending on the environmental factors, among others[49]. Thus,
267 the fact that complete extracts showed stronger inhibitions in surface hydrophobicity and
268 biofilm formation compared with isolated fractions reinforces the theory that the antimicrobial
269 activity of cranberry extracts is a synergistic effect of various phenolic compounds, many of
270 which are probably still unidentified.

271 **CONCLUSIONS**

272 In conclusion, the present work showed that semipreparative-HPLC proved to be a
273 powerful tool for the fractionation of phenolic compounds from complex matrices like cranberry
274 extracts. The results suggested that apart from PACs, other compounds, mainly flavonoids, can
275 act against uropathogenic *E. coli* biofilm formation and also modifying UPEC surface
276 hydrophobicity *in vitro*, one of the first steps of adhesion. Additionally, a synergism between
277 compounds could affect the antibacterial effects of the studied extracts. However, further studies
278 *in vivo* are necessary to confirm their antibacterial activity.

279 **EXPERIMENTAL**

280 **General Experimental Procedures**

281 Formic acid and acetonitrile used for preparing mobile phases were from Sigma-Aldrich
282 (Steinheim, Germany) and Fisher Scientific (Loughborough, Leics, UK), respectively.
283 Ultrapure water with a resistivity value of 18.2 MΩ was obtained from Milli-Q system
284 (Millipore, Bedford, MA, USA). HPLC grade methanol (99.9%) was purchased from Fisher
285 Scientific (Loughborough, Leics, UK). For microbiological determinations, tryptic soy broth

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286 (TSB) (Fluka), phosphate buffered saline pH-7.4 (PBS), ammonium phosphate; acetic acid,
287 methanol, and Hucker's cristal violet were supplied from Sigma-Aldrich (Steinheim, Germany).

288 **Sample preparation**

289 A commercial extract in capsules of American cranberry consisted on concentrated
290 cranberry juice was used to carry out this study (Urell® Pharmatoka, Rueil Malmaison, France).
291 The content of five capsules (200 mg each) was mixed and 5 mg of the cranberry extract were
292 weighted and dissolved in 5 ml of a (50:50, v/v) methanol/water mixture to obtain a final
293 concentration of 1 mg ml^{-1}. Then, the solutions were vortexed for 2 min, sonicated for 10 min,
294 and centrifuged at 984 × g. Finally, the supernatants were filtered through 0.2 µm regenerated
295 cellulose syringe filters. The extraction procedure was carried out in triplicate.

296 For isolation of phenolic compounds from cranberry extract, solution stock at 50 mg ml^{-}
297 ^{1} was prepared by dissolving the appropriate amount of cranberry extract in (50:50, v/v)
298 methanol/water mixture, and the aforementioned procedure was followed.

299 To develop the antimicrobial assays, two solutions of the extract were prepared at 1 mg
300 ml^{-1} (dilution A) and 0.5 mg ml^{-1} (dilution B) in phosphate buffered saline (PBS), pH 7.4.

301 **Isolation of compounds by semipreparative-HPLC**

302 Fractionation was conducted at room temperature using a Gilson semipreparative HPLC
303 system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332),
304 automated liquid handling solutions (model GX-271), and UV-Vis detector (model UV-Vis
305 156). To separate the target compounds, an Ascentis C18 column (10 µm, 250 × 212 mm) was
306 used. The mobile phases consisted of 1% formic acid in water-acetonitrile (90:10, v/v) (phase
307 A) and acetonitrile (phase B). The following optimized multi-step linear gradient was
308 developed: 0 min, 5% B; 10 min, 9.5% B; 35 min, 17.5% B; 50 min, 25% B; 55 min, 100% B;
309 57 min, 5% B; 62 min, 0% B. The initial conditions were held for 10 min. The injection volume
310 was 1 mL. The flow rate used was 15 mL min^{-1}. The separated compounds were monitored with

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311 UV-Vis (220–280 nm). The fraction-collection step consisted of UV-based purification,
312 determining the elution time window for collecting each fraction. Finally, a total of 25 fractions
313 were collected, and the solvent was evaporated under vacuum. The residue of each fraction was
314 weighted and dissolved a) in methanol to obtain a final concentration of 100 ppm to analyze
315 them by HPLC-ESI-MS, and b) in 2 ml of PBS to carry out the antibacterial assays.

316 **Characterization of the fractions by HPLC-ESI-MS**

317 Analyses were carried out by an Agilent 1200 series rapid resolution (Santa Clara, CA,
318 USA) equipped with a binary pump, a vacuum degasser, an autosampler, a thermostated column
319 compartment, and a diode array detector (DAD). Compounds were separated at room
320 temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 × 4.6 mm) (Agilent
321 Technologies, Palo Alto, CA, USA) according to the method proposed by Iswaldi *et al.*[19].

322 The compounds detection was carried out using a Q-TOF mass spectrometer (Agilent
323 6540) equipped with Jet Stream dual electrospray ionization (ESI) interface operating in
324 negative ionization mode. To maintain mass accuracy during the run time, continuous infusion
325 of a reference mass solution containing ions m/z 112.985587 (trifluroacetate anion) and
326 1033.988109 (trifluroacetic adduct of hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or
327 HP-921) was used. Data acquisition in profile mode was governed *via* MassHunter Workstation
328 Software (Agilent Technologies). Data analysis was performed on MassHunter Qualitative
329 Analysis Version B.06.00 (Agilent Technologies).

330 **Bacteria and cultures**

331 A mixture of fourteen strains of uropathogenic *E. coli* (UPEC) were used, ten obtained
332 from patients with acute pyelonephritis (471, 787, 753, 472, 595, 760, 695, 697, 629, and 795),
333 together with four strains obtained from the Spanish Type Culture Collection (CECT): CECT
334 424 (F- thr- leu- lacY mtl- thi- ara gal ton 2 malA xyl, resistant to phages T1, T2, and T6.),
335 CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis), CECT 417

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336 (SupE44 (am). mutant tRNA), and CECT 743 (Serovar. O142 K86B:H6, isolated from children
337 with diarrhea).

338 **Biofilm formation and surface hydrophobicity**

339 To determine the adherence and subsequent biofilm formation of tested mixture of
340 UPEC, a tube test proposed by Stepanovic *et al.*[50] was performed. Briefly, the mixture of
341 uropathogenic strains were subcultured at 37ºC for 24 h in glass tubes with 2.5 mL of tryptic
342 soy broth (TSB). Then, 0.5 mL of the aforementioned culture and 50 µL of the cranberry extract
343 and each selected fraction at two different concentrations displayed in Table S2 (supplementary
344 information) were placed into Eppendorf tubes. An Eppendorf tube without inoculums
345 containing the same amount of TSB was used as a negative control, while 0.5 mL of the
346 bacterial suspension in an Eppendorf tube together with 50 µL of phosphate buffer saline (PBS)
347 was used as a positive control. After incubating for 24 h, the content of each tube was aspirated
348 carefully and washed three times with 1 mL of PBS. Tubes were air dried and 200 µL of 99%
349 methanol were added as a fixative. After 15 min, the excess of methanol was removed and the
350 tubes were air dried. Then, 200 µL of the colorant Hucker's cristal violet solution (2% dye
351 content) were added, and after 5 min the tubes were submerged in distillate water to take out the
352 surplus. After air drying, biofilm was dissolved in each tube with 1 mL 33% acetic acid. Once
353 the absorbance was measured at 570 nm using Boehringer–Mannheim photometer-4010 model
354 (Boehringer GmbH, Mannheim, Germany), results were calculated according to Eq. (1), where
355 OD is the optical density of the strains incubated with the cranberry extract or with each
356 phenolic fraction and ODc is the optical density from the strains after incubating with the same
357 volume of PBS. A scheme describing the assay is displayed in Figure S1 (supplementary
358 information).

359 (1) Δ biofilm = OD/ODc

360 In order to determine the surface hydrophobicity, the ammonium sulphate aggregation
361 test, described by Lindahl *et al.*[51], was carried out. In brief, a mixture of strains was performed
362 in 2 mL of TSB medium. The culture was washed three times with PBS and centrifuged at 562

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363 × g for 10 minutes. Bacteria were resuspended into 0.002 mol L^{-1} sodium phosphate (OD1 at
364 540 nm). Then, 10 µL of the cranberry extract and each selected fraction at two different
365 concentrations displayed in Table S2 (supplementary information) were incubated at room
366 temperature for 30 min in a rotary shaker (Heidolph Reax, ConThermo GmH & Co. KG,
367 Germany) with 100 µL of the bacterial suspension of the selected strains, in PBS. Several
368 solutions of ammonium sulphate at osmolarities ranged from 0.2 to 4 mol L^{-1} in sodium
369 phosphate 0.002 mol L^{-1} were prepared. Then, 10 µL of bacterial suspension with the same
370 volume of ammonium sulphate were added on a slide. The lowest concentration of ammonium
371 sulphate which produced visible aggregation after 30 seconds gentle manual rotation at room
372 temperature was written down. Aggregation with 4 mol L^{-1} solution was interpreted as 0%
373 hydrophobicity, while aggregation with 0.2 mol L^{-1} was interpreted as 95% hydrophobicity. The
374 results obtained, expressed as % hydrophobicity, were calculated according to Eq. (2) where ΔH
375 is the ratio of the hydrophobicity of the strains incubated with the whole extract or with each
376 phenolic fraction and ΔHc are the hydrophobicity of the strains after incubation with an equal
377 volume of PBS. A scheme describing the assay is displayed in Figure S2 (supplementary
378 information).

379 (2) $\% \text{ hydrophobicity} = \Delta H / \Delta Hc * 100$

380 **Statistical analysis**

381 Data of bioactivity are expressed as mean ± standard deviation. Significant differences
382 in the adherence and surface hydrophobicity of *E. coli* pre and post- incubated with the extract
383 or phenolic fractions were determined using the Wilcoxon matched pairs signed rank test by
384 IBM SPSS Statistics (Chicago, IL, USA). Differences between means were considered to be
385 significant when the p value was below 0.05. In addition, Kendall's correlation coefficients of
386 inter-variable concordance were calculated.

387 **Conflicts of interest**

388 The authors declare no competing financial interest.

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389 **Acknowledgements**

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397

398 **Figure captions**

399 **Figure 1.** Semipreparative-HPLC-UV chromatograms of cranberry extract indicating the
400 collected fractions.

401 **Figure 2.** HPLC-MS chromatograms of the isolated fractions from cranberry extract.

402 **Figure 3.** (a) Mean and standard deviation in biofilm formation after incubating *E. coli* strains
403 with each selected fraction; (b) Mean and standard deviations of surface hydrophobicity after
404 incubating *E. coli* strains with each selected fraction. *Significant differences between control
405 group and tested fraction ($p < 0.05$).

406 **Figure 4.** (a) Mean and standard deviation in biofilm formation after incubating *E. coli* strains
407 with cranberry extract; (b) Mean and standard deviations of surface hydrophobicity after
408 incubating *E. coli* strains with cranberry extract. *Significant differences between control group
409 and tested extract ($p < 0.05$).

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Table 1. Retention time and mass spectral data of the compounds characterized in the fractions from cranberry extract by HPLC-ESI-MS in negative mode. *Compounds identified with standard.

Proposed compound	Retention time (min)	Molecular Formula	Calculated m/z ($[M-H]^-$)	Fractions
Quinic acid	5.212	$C_7H_{12}O_6$	191.0561	1,2
Kaempferol arabinoside	5.527	$C_{20}H_{18}O_{10}$	417.0827	1
Procyanidin B	5.736	$C_{30}H_{26}O_{12}$	577.1351	3
Caffeic acid glucoside	6.588	$C_{15}H_{18}O_9$	341.0878	1
Cinnamtannin B1 isomer 1	7.130	$C_{45}H_{36}O_{18}$	863.1829	1,4
Myricetin arabinoside	7.421	$C_{20}H_{18}O_{12}$	449.0725	5
Catechin *	7.765	$C_{15}H_{14}O_6$	289.0718	4

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Procyanidin C1	9.689	$C_{45}H_{38}O_{18}$	865.1985	4
Myricetin glucoside isomer 1	9.065	$C_{21}H_{20}O_{13}$	479.0831	6
Myricetin glucoside isomer 2	9.123	$C_{21}H_{20}O_{13}$	479.0831	7
Dihydroferulic acid glucuronide	9.183	$C_{16}H_{20}O_{10}$	371.0984	4,8
Procyanidin A dimer isomer 1	10.611	$C_{30}H_{24}O_{12}$	575.1195	9
Quercetin glucoside isomer 1	12.155	$C_{21}H_{20}O_{12}$	463.0882	12
Quercetin glucoside isomer 2	12.191	$C_{21}H_{20}O_{12}$	463.0882	11
Procyanidin A dimer isomer 2	12.973	$C_{30}H_{24}O_{12}$	575.1195	12,13
Quercetin-3-O-glucoside *	14.775	$C_{21}H_{20}O_{12}$	463.0882	10
Cinnamtannin B1 isomer 2	15.019	$C_{45}H_{36}O_{18}$	863.1829	14
Quercetin glucoside isomer 3	15.095	$C_{21}H_{20}O_{12}$	463.0882	10
Quercetin arabinoside isomer 1	15.202	$C_{20}H_{18}O_{11}$	433.0776	14, 16
Quercitrin isomer 1	15.663	$C_{21}H_{20}O_{11}$	447.0933	17,18
Quercetin arabinoside isomer 2	16.013	$C_{20}H_{18}O_{11}$	433.0776	15
Myricetin *	20.229	$C_{15}H_{10}O_{8}$	317.0303	21
Quercitrin *	20.847	$C_{21}H_{20}O_{11}$	447.0933	19,20,21
Quercitrin isomer 2	21.668	$C_{21}H_{20}O_{11}$	447.0933	22
Prodelphinidin B	24.246	$C_{30}H_{26}O_{14}$	609.1250	23,24
Quercetin	26.560	$C_{15}H_{10}O_{7}$	301.0354	25

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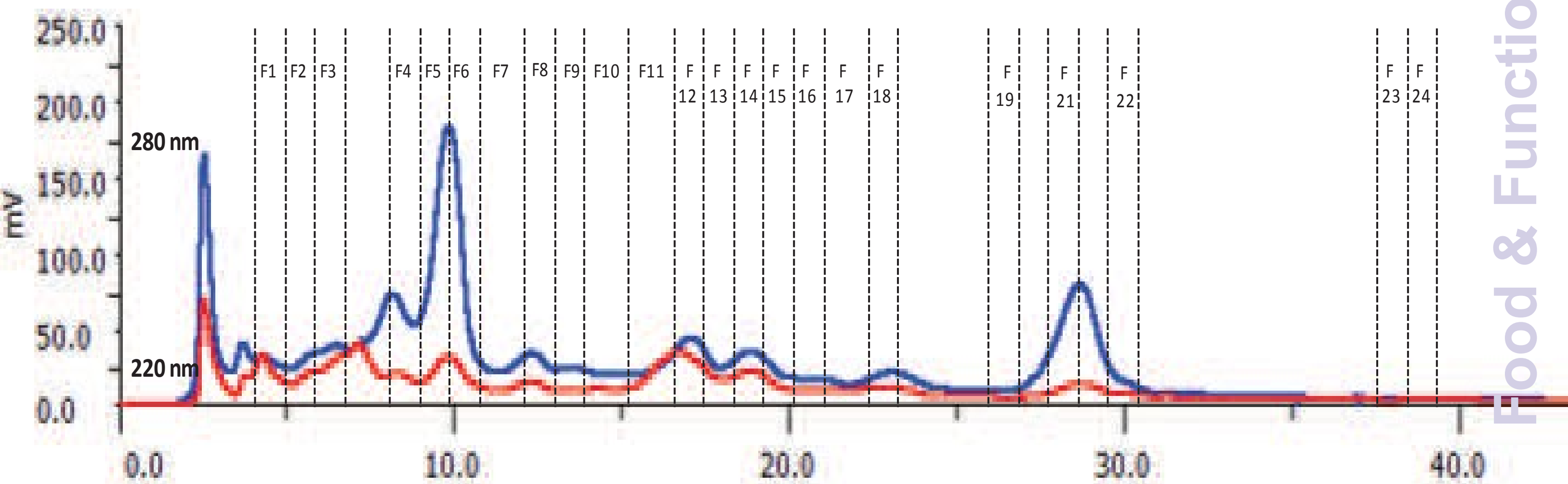

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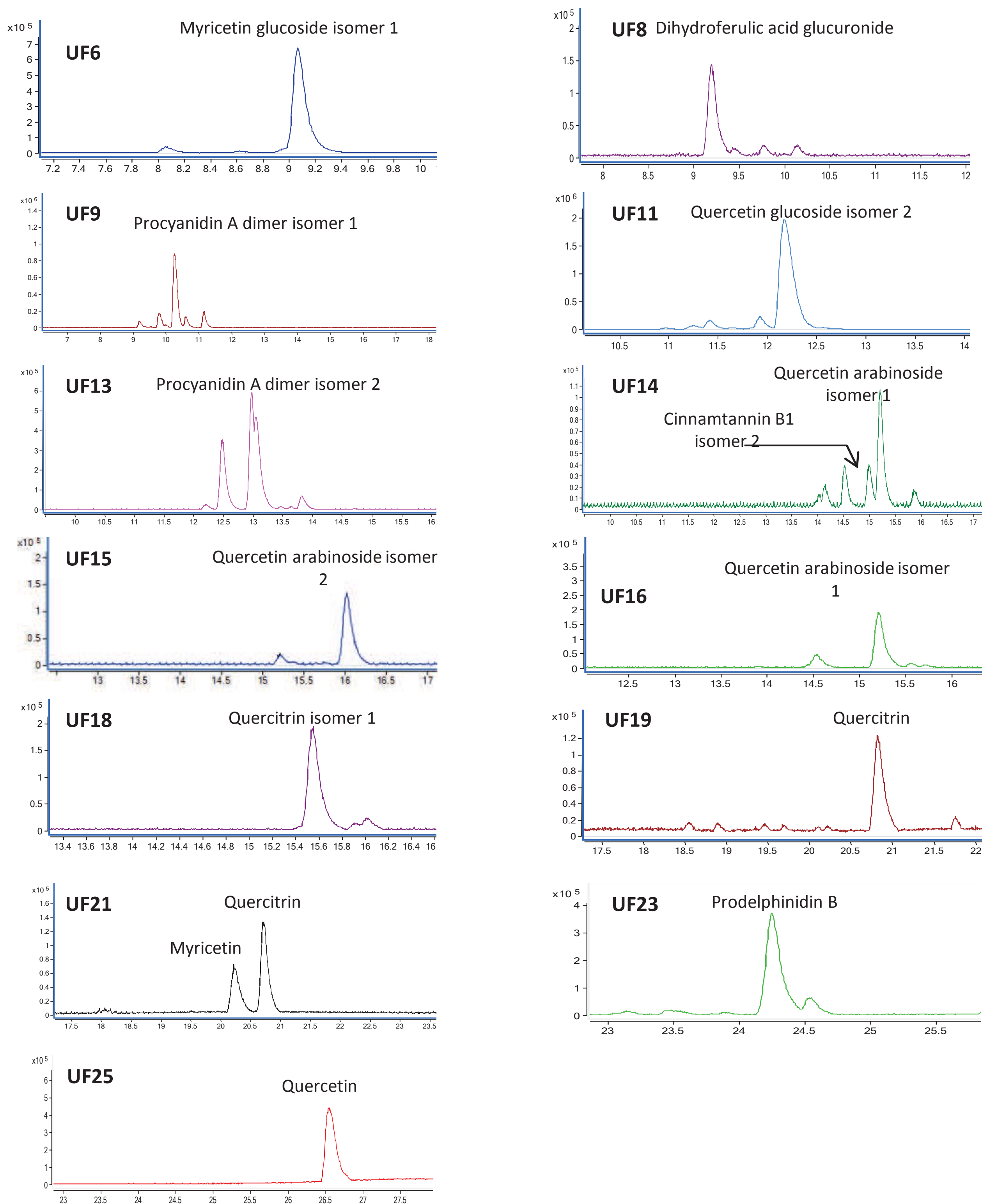
Figure 2



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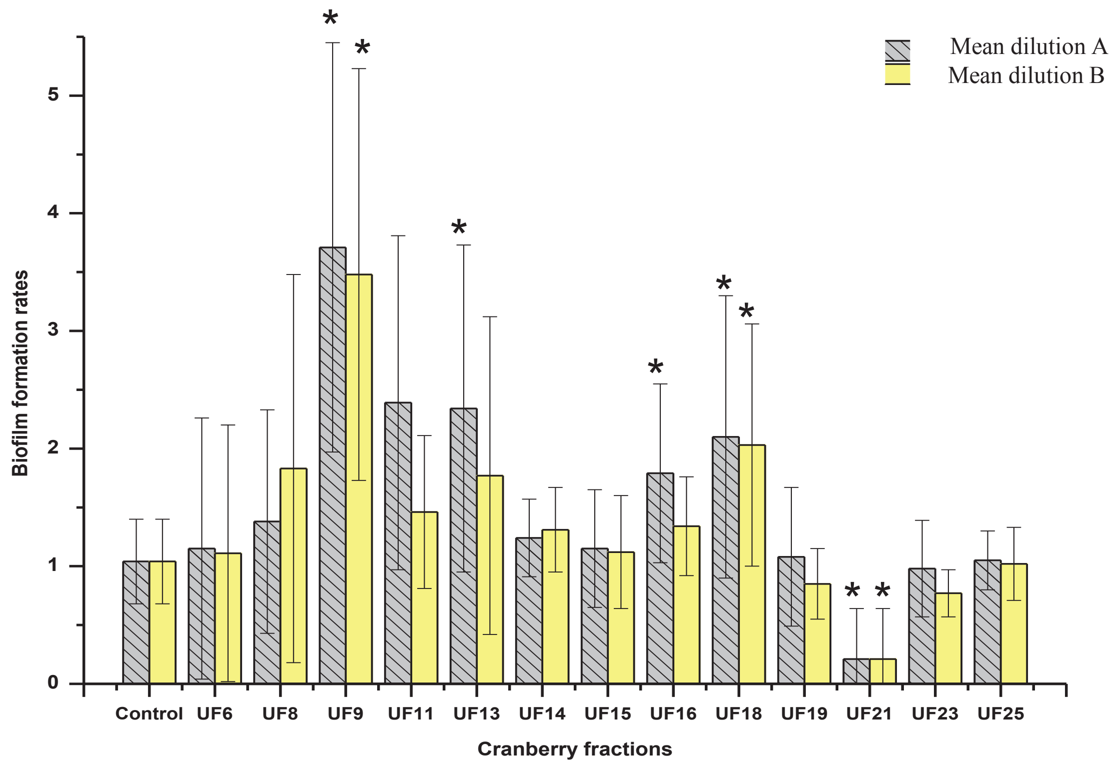

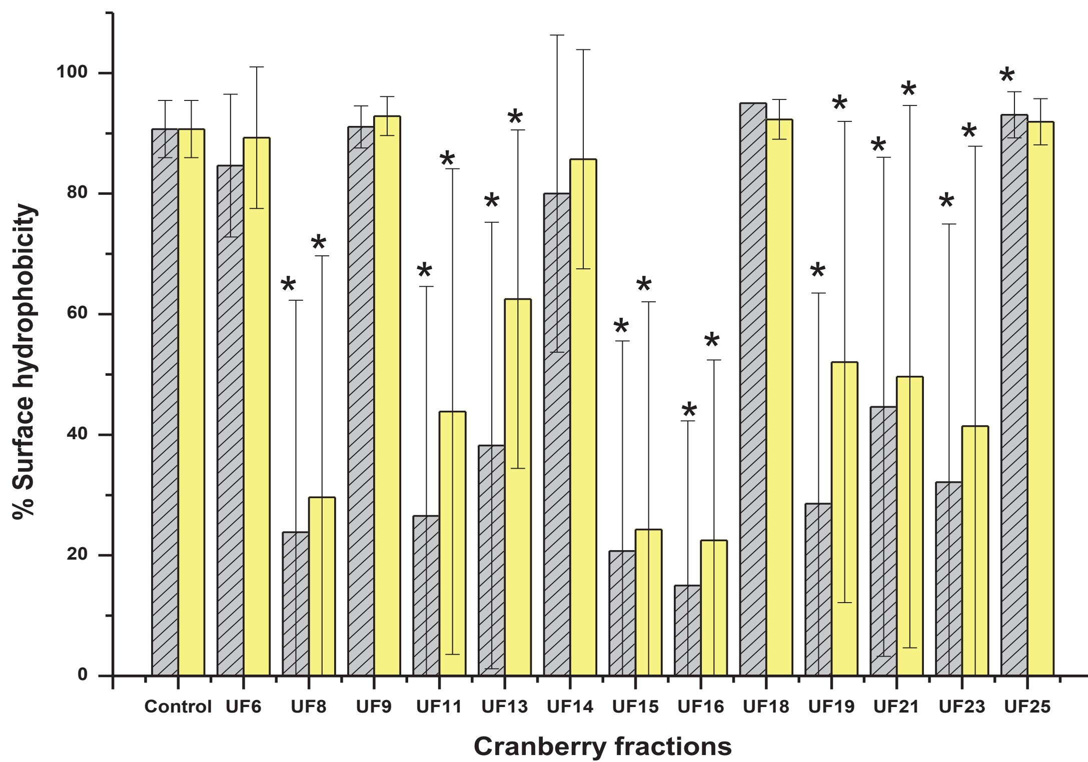

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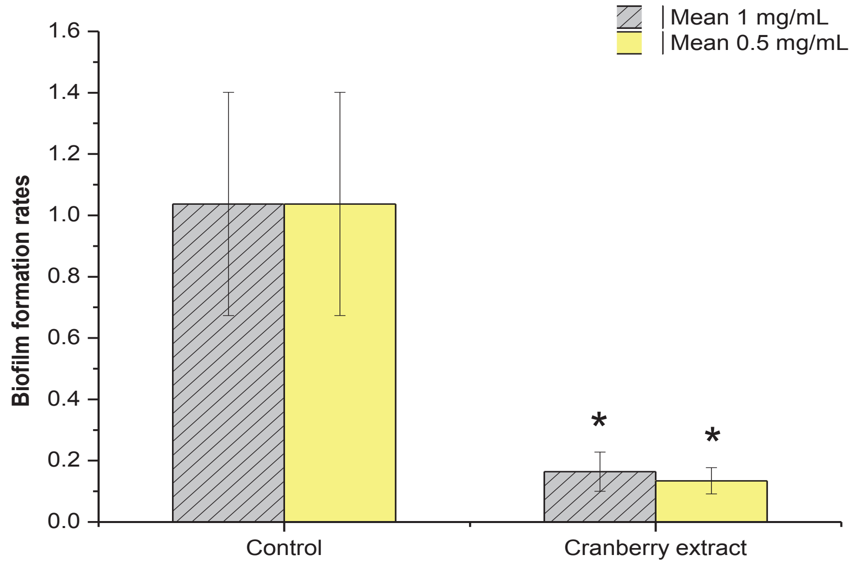

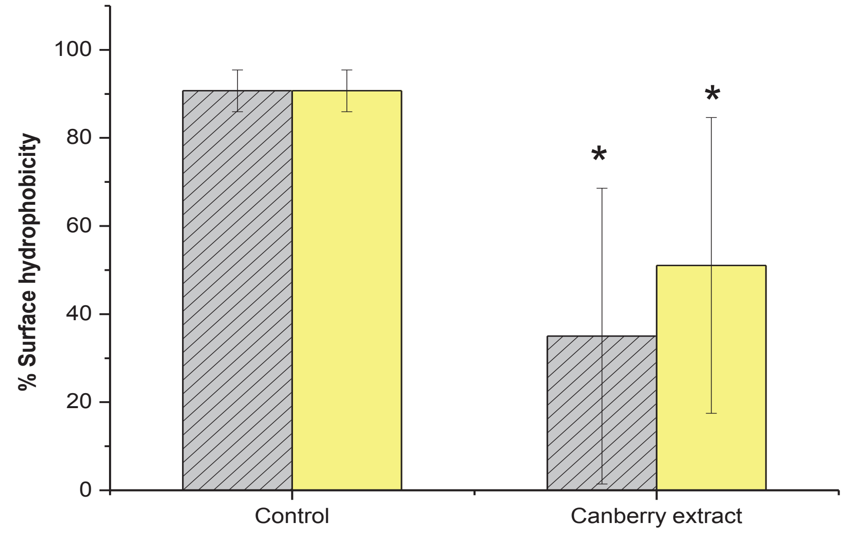

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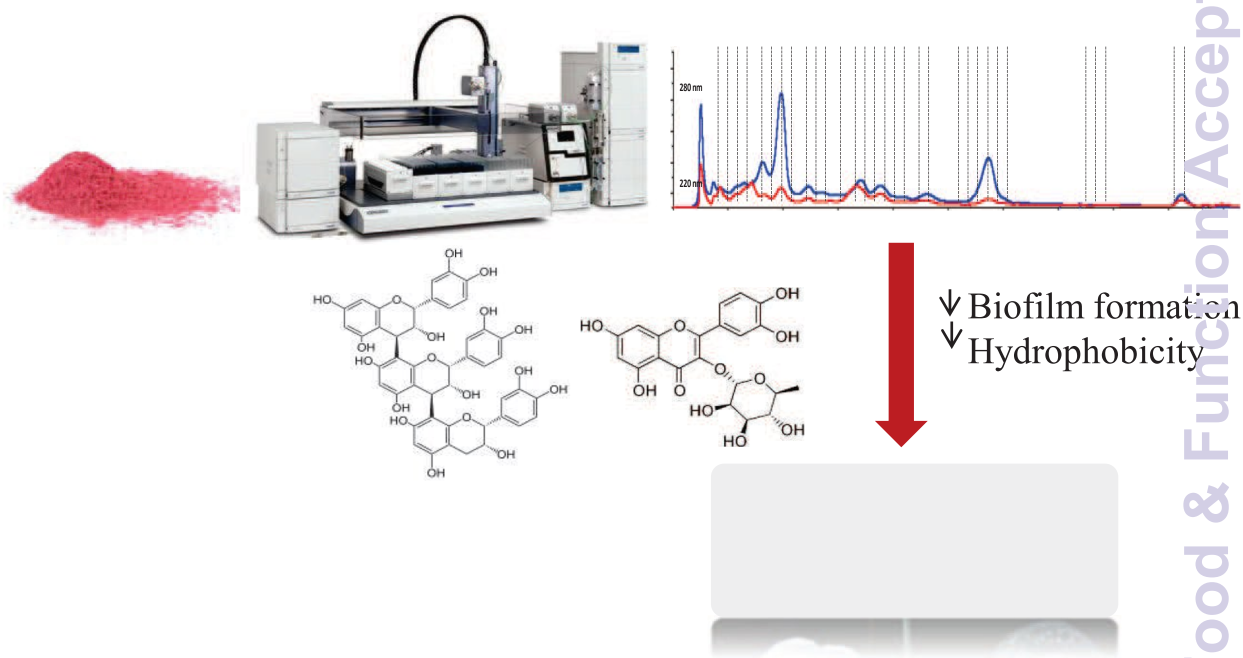

Apart from proanthocyanidins, isolated polyphenols from cranberry can act against *E. coli* adherence and/or modifying its surface hydrophobicity *in vitro*.

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