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

Celia Rodríguez-Pérez [a,b], Rosa Quirantes-Piné [b], José Uberos [c], Cecilia Jiménez-Sánchez [a,b] Alejandro Peña [d] and Antonio Segura-Carretero [a,b].

[a] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain*

[b] *Research and Development Functional Food Center (CIDAF), Health Science Technological Park, Avenida del Conocimiento 37, Edificio BioRegión, 18016 Granada, Spain*

[c] *Unit of Clinical Pediatrics, San Cecilio University Hospital, 18012 Granada, Spain*

[d] *Microbiology Department, San Cecilio University Hospital, Instituto Biosanitario de Investigación (IBIG), Granada, Spain*

Corresponding author: Dr. R. Quirantes Piné, Research and Development Functional Food Center (CIDAF), Health Science Technological Park, Avenida del Conocimiento 37, Edificio BioRegión, 18016 Granada, Spain

E-mail: rquirantes@ugr.es

Fax: +34 958 637083

Abstract

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

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

INTRODUCTION

Cranberries (*Vaccinium macrocarpon*) are popularly consumed as part of the human diet both fresh and processed forms. Additionally, their derived extracts are also used, mainly as part of some botanical dietary supplements forms due to their renowned human health benefits[1]. Cranberry has proved to be an excellent source of bioactive compounds such as flavonoids (procyanidins, flavonols), and phenolic acids derivatives[2]. Thanks to these health-promoting compounds, cranberry and cranberry-based products consumption has been correlated with recurrent urinary tract infections (UTIs) prophylaxis[3,4]. UTI has been defined as the presence of significant number of pathogenic bacteria or organisms in the urinary system and it is considered the most common type of infection in the body, which affects women in a greater extent than men[5] (katouli, M. 2010). *Escherichia coli* (*E. coli*) is the main responsible bacterial species for the appearance of this infection, and causes more than 80 percent of all acquired UTIs in the community[6]. Concretely, the ability of urophatogenic *Escherichia coli* (UPEC) to form biofilm has been strongly associated with recurrent UTIs (Tapiainen, T. et al. 2012; Flores-Mireles, A.L. et al. 2015) and there have been proven that surface hydrophobicity is conductive to adhesion to surfaces and to penetration of host tissues (Krasowska, A. & Sigler, K. 2014) since bacteria have developed many different ways to use hydrophobic effect in order to adhere to substrata, such as previously described by Doyle *et al.*[25]. The importance of biofilms in public health is related to the decreased susceptibility to antimicrobial agents that biofilm-associated microorganisms exhibit. This is the case of *E. coli* which has shown to be increasingly resistant to some of the antibiotics currently used in the treatment of UTIs [8,9]. In addition, the public interest in herbal medicines and natural products is still growing. For this reason, researchers have concluded the re-evaluation of first and second-line therapies for the treatment of UTIs becomes to be pivotal[10]. Consequently, the antimicrobial effect of cranberry products and their phenolic compounds have been widely studied, especially to develop new healthy food ingredients, functional foods, nutraceuticals, and pharmaceuticals[7]. The most accepted theory about the mechanism of action of cranberry compounds for the promotion of

urinary tract health is based on the effects of fructose and PACs in inhibiting the adherence of type 1 and P fimbriae of *E. coli* to the uroepithelial cell receptors[11,12]. Without adhesion, the bacteria cannot infect the mucosal surface. Despite a large number of studies highlighted that there are synergisms between different compounds present in cranberry extracts,[14-16] other authors such as Hisano *et al.* concluded that the use of the whole cranberry for UTIs prevention was not scientifically supported, and for that reason, it is pointed out the necessity of research focused on bioactive compounds from cranberry instead of the entire fruit[3]. However, the isolation of simultaneous compounds from cranberry extracts is an arduous task due to its complexity. Reversed-phase semipreparative high performance liquid chromatography (semipreparative-HPLC) has been increasingly used once possesses an interesting target separation ability, great efficiency and high recovery[17], and therefore can be a valuable tool to solve the aforementioned difficulty.

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

RESULTS AND DISCUSSION

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

Natural extracts usually consist of hundreds of compounds, and the isolation of particular components presents unique problems because the methods used to isolate them are based mainly on their polarity. The similarity of some polyphenolic structures makes that compounds elute at similar retention times, making difficult their separation. For that reason, only few studies have focused on the chromatographic methods for the isolation of multiple compounds simultaneously. In this regard, semipreparative-HPLC is a robust, versatile, and usually rapid technique by which compounds can be purified from complex mixtures[18].

In the current research, the analytical HPLC method previously developed for the characterization of phenolic compounds from cranberry extracts[16] was scaled-up to semipreparative-HPLC scale. Different gradients were tested to enhance the separation of the compounds (data not shown), selecting as optimum the method described in "experimental" section. Figure 1 shows the UV chromatogram of the cranberry extract under study acquired with the proposed method, where the fractions collected are indicated according to their elution order.

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

Despite the scarcity of literature on the fractionation of cranberry using semipreparative-HPLC makes difficult to contrast our optimized method with others, and the results could not be comparable, the optimized method allowed obtaining 25 fractions from the cranberry extract (Table 1), which were composed predominantly by procyanidins (PACs) and flavonols. Even though the difficulty in separating and purifying PACs has been previously highlighted[19], the current method allowed isolating some of them, including A-type procyanidin dimmers, an A-type procyanidin trimer (cinnamtannin B1) and a gallocatechin dimer (prodelphinidin). PACs are the most typical compounds characterized in cranberry, noteworthy for their antioxidant activity, although they may also present other pharmacological and medicinal properties such as anti-carcinogenic, anti-inflammatory, and vasodilator (Rodríguez-Pérez et al. 2015). Isolated cranberry flavonols included quercetin derivatives which have been previously demonstrated to have both *in vivo* and *in vitro* antioxidant, anti-inflammatory,

anticancer, and antidiabetic activities (Kawabata, K. et al. 2015). In addition, four myricetin derivatives were characterized. These compounds are also common dietary flavonoids which have demonstrated antioxidant, cytoprotective, antiviral, antimicrobial, anticancer and antiplatelet activities (Davi, K.P. et al. 2015). Apart from these compounds, one hydroxicinnamic acid derivative (dihydroferulic acid glucuronide) was isolated.

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

Antibacterial activity

Although some authors reported that cranberry does not have any effect against Gram-negative bacteria pathogens such as *E. coli*[13], most of the research converges on the fact that berries, and especially cranberry and cranberry-based products, have both *in vitro* and *in vivo* antibacterial activity[7,12,16,19,20,21]. As aforementioned, the most accepted mechanism of action of cranberry focuses primarily on its ability to prevent bacterial binding to host cell surface membrane (Jepson, R. et al. 2013), one of the initial steps in the infection process. This process is initially mediated by the electrostatic charge (characterized by determining its zeta potential) and consequently surface hydrophobicity of microorganisms followed by other factors such as formation of fimbriae and specific adhesins (Otto, K. et al. 2001). Thus, surface physicochemical parameters such as electrostatic charge are then fundamentally important with regard to influencing overall polarity in order to maintain the degree of bacterial surface

hydrophobicity necessary for the bacterial adhesion. Subsequently, adhesion of bacteria to host surfaces is finally a key element in the formation of biofilms that constitutes a protected mode of growth that allows bacteria to survive in hostile environment (Ribet, D. & Cossart, D. 2015). For that reason, the effect of the previously isolated fractions as well as the whole extract on biofilm formation and surface hydrophobicity of fourteen UPECs has been tested as a way to evaluate the individual contribution of every compound to the antibacterial activity.

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

~~Concretely, Schmidt *et al*. found a significant positive correlation between PAC content of different fractions from blueberry and biological activity in anti-adhesion assays[19]~~After testing the selected fractions, two concentrations of F9 made up of procyanidin type-A dimer, showed a statistically significant increase in biofilm formation compared with the control (Fig. 3). Other research has also described an increase of biofilm formation in four of the 20 *E. coli* strains tested after consuming cranberry juice (Tapiainen, T. et al. 2012) and a reduction of biofilm formation only in one of them. However, F9 did not significantly change surface hydrophobicity. On the other hand, F13 (made up of other isomer of procyanidin type-A dimer) at the highest concentration (dilution A) caused an increase in biofilm formation while both concentrations tested significantly decreased surface hydrophobicity. In any case, it should be pointed out that the hydrophobicity of bacteria can vary even within the same strain depending on the mode and stage of growth (Goulter, et al. 2009). Despite the study of PACs in *E. coli* has been widely described, controversial results are still reported in literature. Foo *et al*. also found a weak activity of procyanidin A2 against the inhibition of adherence of *E. coli*[27]. In another study, PACs as a group of compounds inhibited the growth of *E. coli* CM 871, with no inhibition of *E. coli* 50[14]. Foo *et al* also proved the anti-adherent effect of procyanidin trimers.

However, no statistical differences were found between F14 (made up of cinnamtannin B1 and quercetin arabinoside) and the control in both assays tested. Prodelphinidin B (F23) also influenced the antibacterial effect against *E. coli* by decreasing the bacteria surface hydrophobicity. Prodephinidins with pyrogallol groups, which have similar structures to procyanidins except for their hydroxyphenyl group, have reported to have stronger antibacterial activity than procyanidins with the catechol groups (Taguri, T. et al. 2006). However, the different results obtained from different isolated PACs, reinforce the theory proposed by Schmidt *et al.* who concluded that it was likely that a mixture of several high molecular weight PACs were responsible for the anti-proliferation and anti-adhesion activity.

~~Microorganisms act in different ways to adhere due to influence by the substrata, nutrients, ionic strength, pH values, and temperatures, and also by their phenotype and genotype. Thus, the ability of microorganisms to attach to surfaces is crucial for the beginning of colonization[23]. Adhesion is influenced by hydrophobicity and surface charge, among others factors. This means that when the surface hydrophobicity of a bacterial cell is increased, the charge on the cell surface (zeta potential) is reduced[24]. At the same time, bacteria have developed many different ways to use hydrophobic effect in order to adhere to substrata, such as previously described by Doyle *et al.*[25].~~

Regarding isolated flavonols, fraction formed by myricetin and quercitrin (F21) was the most active fraction against the *E. coli* biofilm formation and also influenced the decrease in *E. coli* surface hydrophobicity. Bacterial hydrophobicity has been proved to be largely influenced by the residues and structures on the surface of the cell (Goulter, R.M. et al. 2009). In this way, recent research has pointed out that phytochemicals such as flavonoids can modify bacterial membrane surface hydrophobicity[23] probably based on their ability to complex with extracellular and soluble proteins as well as with bacterial cell walls. Concretely, three mechanisms of action of flavonoids have been proposed: inhibition of nucleic acid synthesis, cytoplasmic membrane damage and inhibition of energy metabolism (Cushnie, T.P. & Lamb, A.J. 2011). Although the anti-adherent effect of myricetin remains controversial, some authors

have found that 0.5 mg mL^{-1} of myricetin strongly inhibited the growth of *E. coli*[14]. Only few studies have been carried out in order to assess the flavonoids structure-antibacterial activity relationship. In this sense, some authors concluded that the hydroxylation at position 5 on the A ring and at position 3 on the C ring improves the antibacterial activity of flavones decreasing membrane fluidity (Smejkal, K. et al. 2008; Li, H.Q. et al. 2009; Wu Ting et al. 2013). These previous results could explain the antibacterial effects that the combination of quercitrin and myricetin (F21) showed in both assays. Cowan *et al.* reported that more lipophilic flavonoids may disrupt microbial membranes[13]. Furthermore, Wojnicz, *et al*. affirmed that flavonoids such as quercetin, reduced biofilm synthesis because they can suppress autoinducer-2 activity, which is responsible for cell-to-cell communication[28]. In particular other authors have described the existence of antibacterial activity of quercetin against *E. coli*[6]. Contrary to these previous findings, F25, formed by pure quercetin, a molecule that has a lipophilic character despite the presence of five hydroxyl groups in its structure, not only did not show statistical differences in UPEC biofilm formation at two tested concentrations, but also significantly increased the UPEC surface hydrophobicity compared with control at the highest concentration tested (dilution A). Some authors affirmed, in base of their results, that the degree of hydroxylation might affect the antimicrobial activity of phenolic compounds, indicating that the more polar flavonoids, the more antibacterial effect[14]. In the current study, this theory could be applicable when comparing F25 (quercetin) and F21 (quercitrin and myricetin). The addition of one more hydroxyl group on the aromatic ring of myricetin compared with quercetin may be responsible for its antimicrobial activity. Other research attributes its antimicrobial mechanism against Gram-negative to a reaction with DNA or inhibition of protein synthesis bacteria[29,30]. An early theory based on that hydrophobic effect may be the primary driving force for the adhesion of most pathogens was also proposed[31]. However, taking into account the abovementioned case of quercetin, no relation was observed between *E. coli* surface hydrophobicity and biofilm formation rates.

Despite the great general interest in glycosylated flavonoids due to their diverse bioactivity (Xiao, J. 2014), research focused on their antibacterial properties is still at the

developmental stage. None of the tested concentrations of F6 (myricetin glucoside) showed any activity against biofilm formation nor modifying surface hydrophobicity. Some authors have pointed out that the glycosylation of flavonoids leads to a loss of activity against some Gram-negative bacteria (Xu, Hong-Xi & Lee, Song F. 2001). In addition, early studies concluded that quercetin monosaccharide derivatives showed weak activity against *E. coli* (Bernard, F.X. et al. 1997). Following with these compounds, other plant extracts such as white garlic extract, which contains a high concentration of quercetin-4-O-glucoside and quercetin-3,4-O-diglucoside, had a large inhibiting activity on the growth of *E. coli*, among other Gram-negative bacteria[6]. The current results show that quercetin derivatives do not always produce the same antibacterial effect. On one hand, fractions 18 and 19, made up of quercitrin isomer and quercitrin (quercetin-3- rhamnoside) respectively, showed different antibacterial activity. While incubation with F18 caused a statistically significant increment of UPEC biofilm formation compared with the control and did not present significant differences on surface hydrophobicity, F19 (quercitrin) did not show statistical differences in biofilm formation rates but produced a significant reduction on surface hydrophobicity. Taking into account that F19 was tested at lower concentrations than F18, as depicted in table S2 (supplementary information), this fact suggests that the position of sugar moieties influences the antibacterial activity of flavonoids. Previous studies reported that among quercetin glycosides tested, quercetin-3-rhamnoside exhibited the strongest antibacterial activity against Gram-negative bacteria whereas other quercetin glycosides showed weak or no activity against the same Gram-negative bacteria (K. Waage, S. et al. 1985). On the other hand, F15 and F16, made up of quercetin arabinoside isomers, showed similar trends in significant surface hydrophobicity reduction even testing different concentrations (Table S1, supplementary information) while only F16 at 300 μg mL^{-1} (dilution A) significantly increased the biofilm formation rate.

In addition, both tested concentrations of fraction F8, made up of mainly dihydroferulic acid glucuronide, also showed a reduction in the hydrophobicity of *E. coli*. In this regard, Borges *et al*. found that ferulic acid had antimicrobial activity against *E. coli* by irreversible

changes in membrane properties through hydrophobicity changes that caused local rupture or pore formation in the cell membranes causing the loss of essential intracellular constituents[32]. ~~Panizzi *et al*. also described antimicrobial activity from ferulic acid isolated from *Rubus ulmifolius*[33].~~ Despite Borges *et al*. also concluded in other study that ferulic acid reduced mass of biofilm formed by Gram-negative bacteria (Borges et al. 2012), dihydroferulic acid glucuronide did not show statistically differences compared with the control.

If we look at the whole extract, ~~Figure 4 shows the mean and standard deviation (SD) of biofilm formation and hydrophobicity for UPEC strains after the incubation. Regarding the activity of the extract,~~ the data revealed statistical differences with respect to control in both, biofilm formation and surface hydrophobicity, after incubating UPEC strains with the cranberry extract independent of the concentrations tested (Figure 4). This finding suggests that even at low dosage, cranberry extract presents antibacterial activity *in vitro*. As pointed out along the text, the hydrophobic properties of microbial surfaces are conducive to adhesion and, thus, to penetration of host tissues. Taking into account the capacity of UPEC to form biofilms, it could be expected a positive relationship between hydrophobicity and biofilm formation. However, the nonparametric Kendall's rank correlation disclosed that there was no trend between surface hydrophobicity and adherence (W=0.236; p=0.019) of UPEC tested after the incubation with cranberry extract. These results could be attributed to the different behavior of each strain. In fact, despite most of UPEC strains are *in vitro* positive for biofilm production (Maheswari, U.B. et al. 2013), it has been previously reported that even the same strain can respond very differently to biofilm formation depending on the environmental factors, among others (Reisner, A. et al. 2006). Thus, the fact that complete extracts showed stronger inhibitions in surface hydrophobicity and biofilm formation compared with isolated fractions reinforces the theory that the antimicrobial activity of cranberry extracts is a synergistic effect of various phenolic compounds, many of which are probably still unidentified.

CONCLUSIONS

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

EXPERIMENTAL

General Experimental Procedures

Formic acid and acetonitrile used for preparing mobile phases were from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Loughborough, Leics, UK), respectively. Ultrapure water with a resistivity value of 18.2 MΩ was obtained from Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol (99.9%) was purchased from Fisher Scientific (Loughborough, Leics, UK). For microbiological determinations, tryptic soy broth (TSB) (Fluka), phosphate buffered saline pH-7.4 (PBS), ammonium phosphate; acetic acid, methanol, and Hucker's cristal violet were supplied from Sigma-Aldrich (Steinheim, Germany).

Sample preparation

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

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

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

Isolation of compounds by semipreparative-HPLC

Fractionation was conducted at room temperature using a Gilson semipreparative HPLC system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271), and UV-Vis detector (model UV-Vis 156). To separate the target compounds, an Ascentis C18 column (10 µm, 250 × 212 mm) was used. The mobile phases consisted of 1% formic acid in water-acetonitrile (90:10, v/v) (phase A) and acetonitrile (phase B). The following optimized multi-step linear gradient was developed: 0 min, 5% B; 10 min, 9.5% B; 35 min, 17.5% B; 50 min, 25% B; 55 min, 100% B; 57 min, 5% B; 62 min, 0% B. The initial conditions were held for 10 min. The injection volume was 1 mL. The flow rate used was 15 mL min^{-1}. The separated compounds were monitored with UV-Vis (220–280 nm). The fraction-collection step consisted of UV-based purification, determining the elution time window for collecting each fraction. Finally, a total of 25 fractions were collected, and the solvent was evaporated under vacuum. The residue of each fraction was weighted and dissolved a) in methanol to obtain a final concentration of 100 ppm to analyze them by HPLC-ESI-MS, and b) in 2 ml of PBS to carry out the antibacterial assays.

Characterization of the fractions by HPLC-ESI-MS

Analyses were carried out by an Agilent 1200 series rapid resolution (Santa Clara, CA, USA) equipped with a binary pump, a vacuum degasser, an autosampler, a thermostated column compartment, and a diode array detector (DAD). Compounds were separated at room temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA) according to the method proposed by Iswaldi *et al.*[16].

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

Bacteria and cultures

A mixture of fourteen strains of uropathogenic *E. coli* (UPEC) were used, ten obtained from patients with acute pyelonephritis (471, 787, 753, 472, 595, 760, 695, 697, 629, and 795), together with four strains obtained from the Spanish Type Culture Collection (CECT): CECT 424 (F- thr- leu- lacY mtl- thi- ara gal ton 2 malA xyl, resistant to phages T1, T2, and T6.), CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis), CECT 417 (SupE44 (am). mutant tRNA), and CECT 743 (Serovar. O142 K86B:H6, isolated from children with diarrhea).

Biofilm formation and surface hydrophobicity

To determine the adherence and subsequent biofilm formation of tested mixture of UPEC, a tube test proposed by Stepanovic *et al.*[34] was performed. Briefly, the mixture of uropathogenic strains were subcultured at 37ºC for 24 h in glass tubes with 2.5 mL of tryptic soy broth (TSB). Then, 0.5 mL of the aforementioned culture and 50 µL of the cranberry extract and each selected fraction at two different concentrations displayed in Table S2 (supplementary information) were placed into Eppendorf tubes. An Eppendorf tube without inoculums containing the same amount of TSB was used as a negative control, while 0.5 mL of the bacterial suspension in an Eppendorf tube together with 50 µL of phosphate buffer saline (PBS) was used as a positive control. After incubating for 24 h, the content of each tube was aspirated carefully and washed three times with 1 mL of PBS. Tubes were air dried and 200 µL of 99%

methanol were added as a fixative. After 15 min, the excess of methanol was removed and the tubes were air dried. Then, 200 μL of the colorant Hucker's cristal violet solution (2% dye content) were added, and after 5 min the tubes were submerged in distillate water to take out the surplus. After air drying, biofilm was dissolved in each tube with 1 mL 33% acetic acid. Once the absorbance was measured at 570 nm using Boehringer–Mannheim photometer-4010 model (Boehringer GmbH, Mannheim, Germany), results were calculated according to Eq. (1), where OD is the optical density of the strains incubated with the cranberry extract or with each phenolic fraction and ODc is the optical density from the strains after incubating with the same volume of PBS. A scheme describing the assay is displayed in Figure S1 (supplementary information).

(1) Δ biofilm = OD/ODc

In order to determine the surface hydrophobicity, the ammonium sulphate aggregation test, described by Lindahl *et al.*[35], was carried out. In brief, a mixture of strains was performed in 2 mL of TSB medium. The culture was washed three times with PBS and centrifuged at 562 × g for 10 minutes. Bacteria were resuspended into 0.002 mol L^{-1} sodium phosphate (OD1 at 540 nm). Then, 10 μL of the cranberry extract and each selected fraction at two different concentrations displayed in Table S2 (supplementary information) were incubated at room temperature for 30 min in a rotary shaker (Heidolph Reax, ConThermo GmH & Co. KG, Germany) with 100 μL of the bacterial suspension of the selected strains, in PBS. Several solutions of ammonium sulphate at osmolarities ranged from 0.2 to 4 mol L^{-1} in sodium phosphate 0.002 mol L^{-1} were prepared. Then, 10 μL of bacterial suspension with the same volume of ammonium sulphate were added on a slide. The lowest concentration of ammonium sulphate which produced visible aggregation after 30 seconds gentle manual rotation at room temperature was written down. Aggregation with 4 mol L^{-1} solution was interpreted as 0% hydrophobicity, while aggregation with 0.2 mol L^{-1} was interpreted as 95% hydrophobicity. The results obtained, expressed as % hydrophobicity, were calculated according to Eq. (2) where ΔH is the ratio of the hydrophobicity of the strains incubated with the whole extract or with each

phenolic fraction and ΔHc are the hydrophobicity of the strains after incubation with an equal volume of PBS. A scheme describing the assay is displayed in Figure S2 (supplementary information).

(2) % hydrophobicity = ΔH/ ΔHc * 100

Statistical analysis

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

Conflicts of interest

The authors declare no competing financial interest.

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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
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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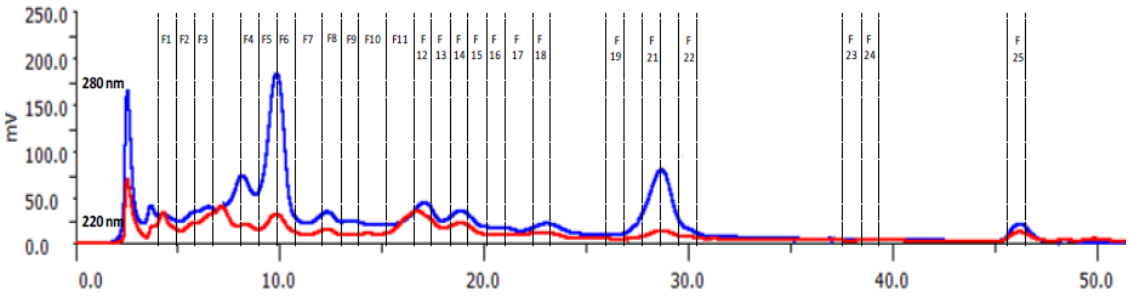


Figure 1. Semipreparative-HPLC-UV chromatograms of cranberry extract indicating the collected fractions.

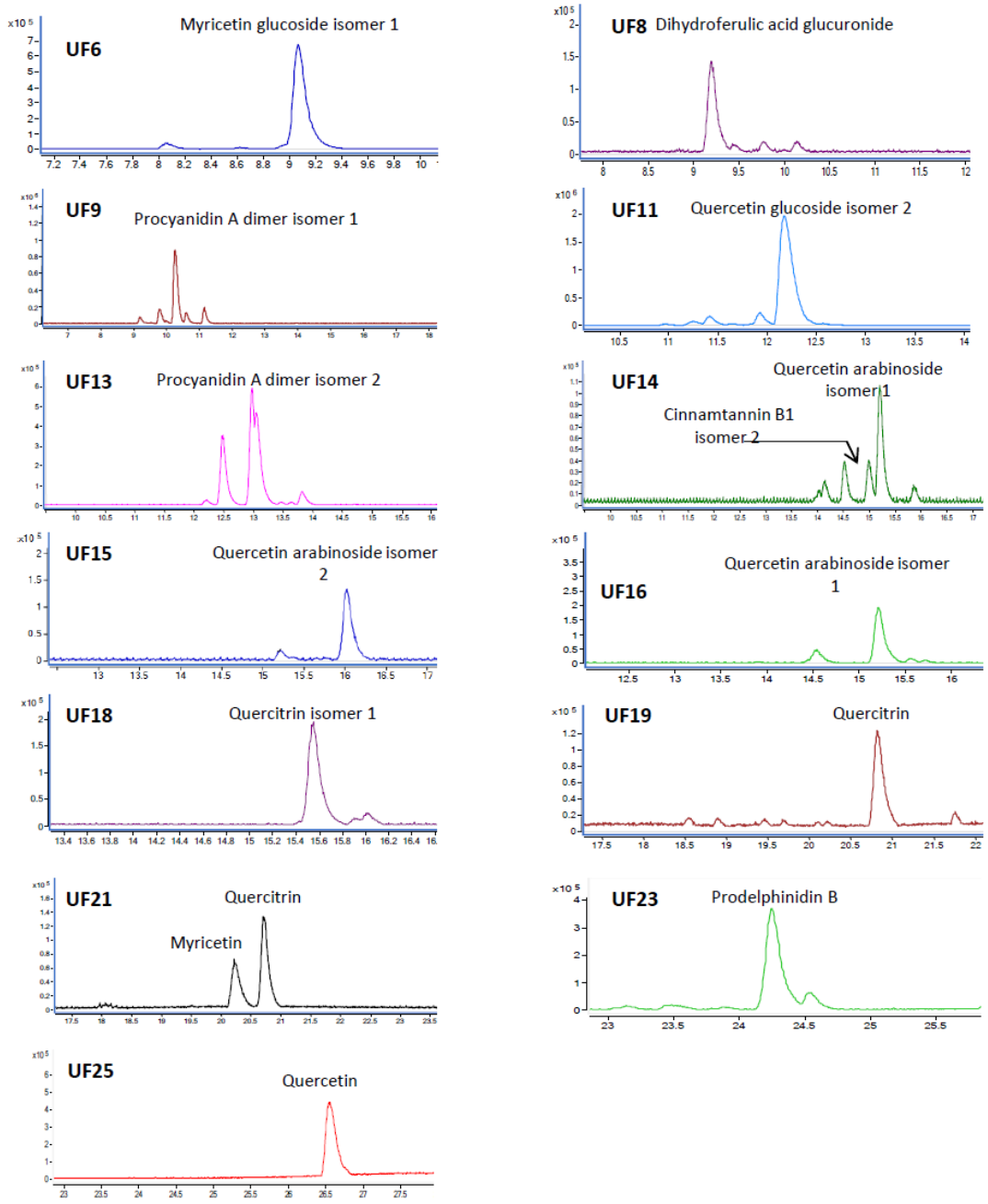
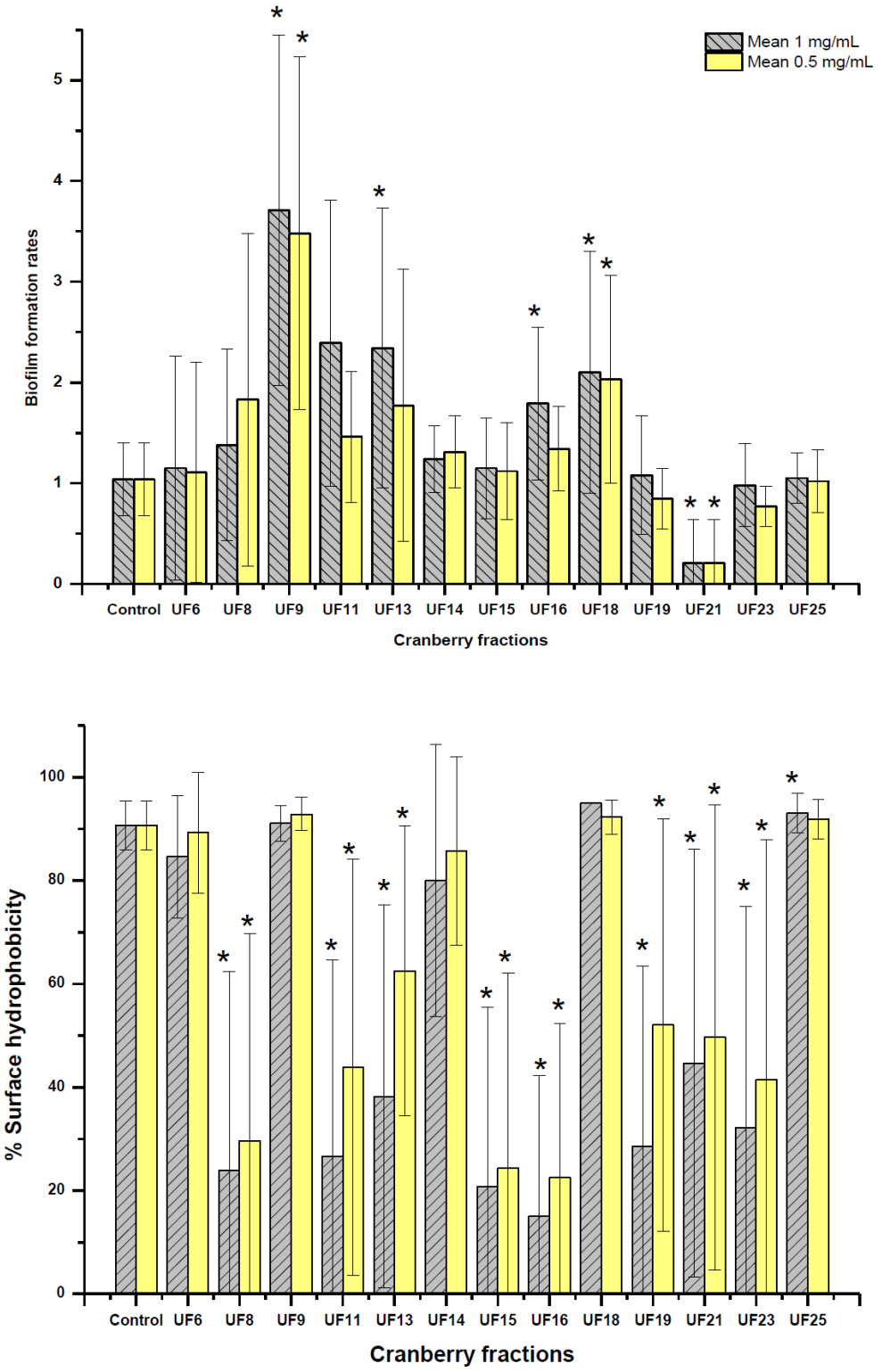
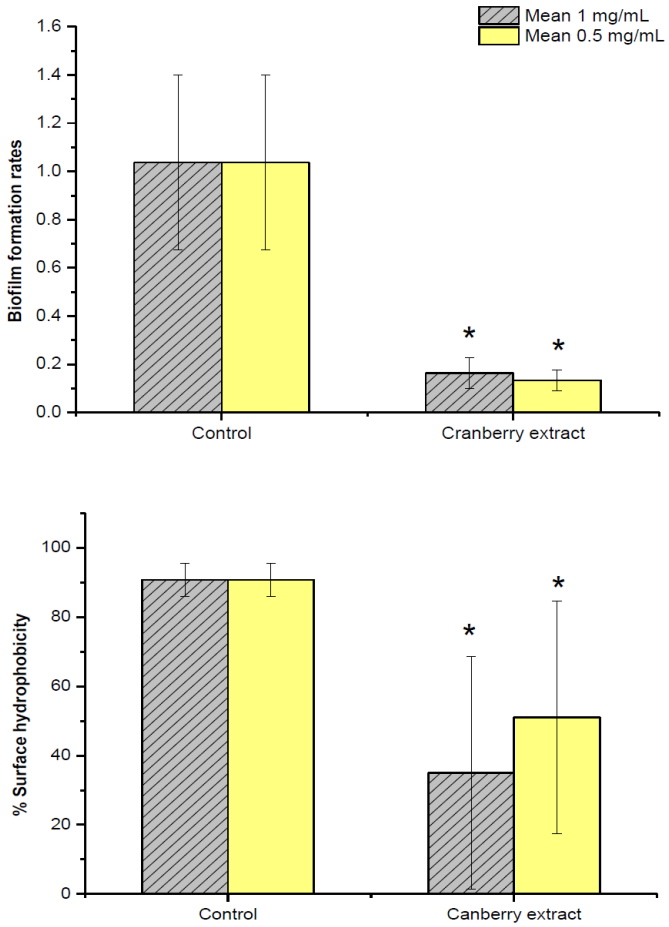


Figure 2. HPLC-MS chromatograms of the isolated fractions from cranberry extract.



489

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



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

499

Table S1. Surface hydrophobicity and biofilm formation rates after incubating E. coli with each cranberry fraction compared with control. Dil A, dilution at 1 mg mL-1; Dil B, dilution at 0.5 mg mL-1. *Significant differences between control group and tested extract ($p < 0.05$).

Hydrophobicity	z	Asymptotic significance	Biofilm formation	z	Asymptotic significance
Extract Dil A	-3.063	0.002*	Extract Dil A	- 3.296	0.001*
Extract Dil B	-3.065	0.002*	Extract Dil B	- 3.296	0.001*
F6 Dil A	-1.913	0.056	F6 Dil A	-0.795	0.427
F6 Dil B	-0.577	0.564	F6 Dil B	-0.852	0.394
F8 Dil A	-3.083	0.002*	F8 Dil A	-0.795	0.427
F8 Dil B	-3.081	0.002*	F8 Dil B	-1.931	0.053
F9 Dil A	-0.122	0.903	F9 Dil A	-3.408	0.001*
F9 Dil B	-1.294	0.196	F9 Dil B	-3.296	0.001*
F11 Dil A	-3.117	0.002*	F11 Dil A	-0.659	0.510
F11 Dil B	-2.988	0.003*	F11 Dil B	-0.471	0.638
F13 Dil A	-3.113	0.002*	F13 Dil A	-3.124	0.002*
F13 Dil B	-2.671	0.008*	F13 Dil B	-1.704	0.088
F14 Dil A	-1.256	0.209	F14 Dil A	-1.590	0.112
F14 Dil B	-0.723	0.470	F14 Dil B	-1.533	0.125
F15 Dil A	-3.074	0.002*	F15 Dil A	-0.738	0.460
F15 Dil B	-3.074	0.002*	F15 Dil B	-0.454	0.650
F16 Dil A	-3.315	0.001*	F16 Dil A	-2.556	0.011*
F16 Dil B	-3.188	0.001*	F16 Dil B	-1.533	0.125
F18 Dil A	-2.456	0.014*	F18 Dil A	-2.668	0.008*
F18 Dil B	-0.586	0.558	F18 Dil B	-2.731	0.006*
F19 Dil A	-3.237	0.001*	F19 Dil A	-0.284	0.776
F19 Dil B	-2.989	0.003*	F19 Dil B	-0.966	0.334
F21 Dil A	-2.849	0.004*	F21 Dil A	-2.840	0.005*
F21 Dil B	-2.673	0.008*	F21 Dil B	-3.067	0.002*
F23 Dil A	-2.833	0.005*	F23 Dil A	0.00	1.00
F23 Dil B	-2.631	0.009*	F23 Dil B	-1.420	0.156
F25 Dil A	-2.449	0.014*	F25 Dil A	-0.454	0.650
F25 Dil B	-0.791	0.429	F25 Dil B	-0.284	0.776

Table S2. Concentration tested in bioactivity assays

Fractions tested	Dilution A (μg mL^{-1})	Dilution B (μg mL^{-1})
F6	550	275
F8	550	275
F9	450	225
F11	850	425
F13	750	375
F14	500	250
F15	450	225
F16	350	175
F18	550	275
F19	300	150
F21	350	175
F23	250	125
F25	300	150

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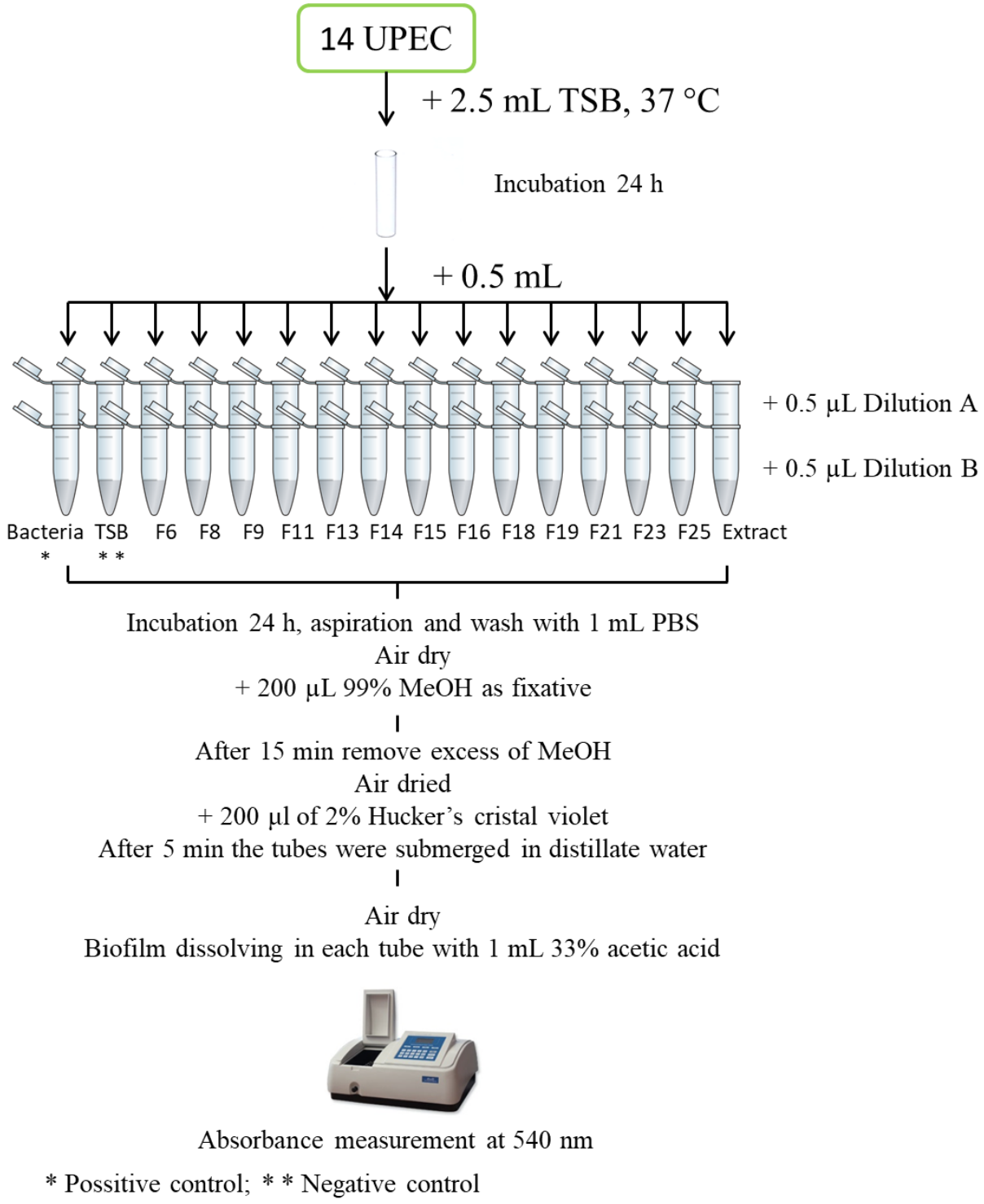

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507 **Figure S1.** Scheme describing the adherence and subsequent biofilm formation assay.

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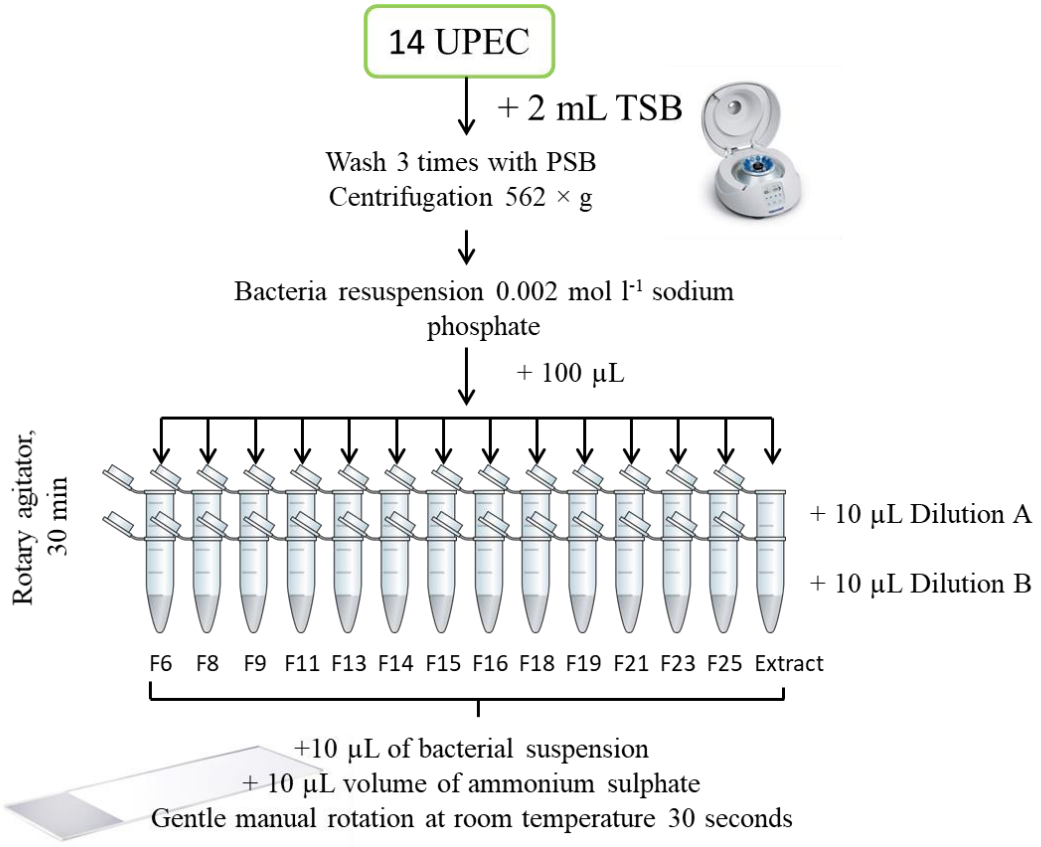

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510 **Figure S2.** Scheme describing the surface hydrophobicity assay based on the ammonium

511 sulphate aggregation test.

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513