Profiling *Vaccinium macrocarpon* components and metabolites in human urine and the urine ex-vivo effect on *Candida albicans* adhesion and biofilm-formation

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**ABSTRACT**

The aim of this work was to profile, by using an HPLC-MS/MS method, cranberry compounds and metabolites found in human urine after ingestion of a highly standardized cranberry extract (Anthocran®). Two different strategies were adopted for the data analysis: a targeted and an untargeted approach. These strategies allowed the identification of 42 analytes including cranberry components, known metabolites and metabolites hitherto unreported in the literature, including six valerolactones/valeric acid derivatives whose presence in urine after cranberry consumption has never been described before. Absolute concentrations of 26 over 42 metabolites were obtained by using pure available standards. Urine collected at different time points after the last dosage of Anthocran® were tested on the reference strain *C. albicans* SC5314, a biofilm-forming strain. Fractions collected after 12 h were found to significantly reduce the adhesion and biofilm formation compared to the control (p < 0.05). A similar effect was then obtained by using Anthocran™ Phytosome™, the lecithin formulation containing 1/3 of standardized cranberry extract and formulated to enhance the absorption of the cranberry components. The urinary profile of cranberry components and metabolites in the urine fractions collected at 1 h, 6 h and 12 h after the last capsule intake were then reproduced by using the pure standards at the concentration ranges found in the urine fraction, and tested on *C. albicans*. Only the mixture mimicking the urinary fraction collected at 12 h and containing as main components, quercetin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was found effective thus confirming the ex-vivo results.

1. Introduction

*Candida albicans* is one of the most common fungi causing disease in humans and the most frequently isolated fungal pathogen in nosocomial urinary tract infections (UTIs) [1,2]. Urological devices, urological procedures, diabetes and being female are the main factors linked to candiduria [3]. Catheters, which are used in up to 20% of hospitalized subjects [4], represent an adhesion substrate for microorganisms that can easily develop biofilm on plastic or silicone surfaces. The most important feature of microbial biofilms is their tolerance to antimicrobial therapies [5], leading to recurrent or persistent infections. Therefore, alternative approaches to conventional antifungal therapy are desirable and among these the search of botanical products provides opportunities for new therapeutic approaches.

Cranberry (*Vaccinium macrocarpon*) is a rich source of polyphenols, which possess beneficial properties towards pathogenic infections including urinary tract infections (UTIs), dental caries and stomach ulcers [6]. Moreover, berry phenolics showed antioxidant, anti-inflammatory and anticancer properties [7,8]. A synergy of all the phytochemicals could explain the great health benefits of cranberry reported in *in vitro*
2. Materials and methods

2.1. Reagents

Formic acid, ethyl gallate, protocatechuic acid, p-coumaric acid, gallic acid, sinapinic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 3-(4-hydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, 3,4-dihydroxyhydrocinnamic acid, 2-hydroxyhippuric acid, quinic acid, 2-methylhippuric acid, YPD medium, Roswell Park Memorial Institute 1640 medium (RPMI), phosphate buffered saline (PBS), crystal violet, methanol and LC–MS grade solvents were purchased from Merck KGaA, Darmstadt, Germany. Phosphate buffered saline (PBS), crystal violet, methanol and LC–MS grade solvents were purchased from Merck KGaA, Darmstadt, Germany. Quercetin, lecithin formulation of the standardized cranberry extract was purchased from Indena S.p.A (Milan, Italy). 12 mg PACs/capsule Anthocran™ Phytosome™ and placebo capsule were supplied by Indena S.p.A (Milan, Italy).

2.2. Synthesis of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (I)

1H NMR spectra were recorded operating at 300 MHz while 13C NMR at 75.43 MHz. Chemical shifts are reported in ppm relative to TMS as internal standard. Signal multiplicities are assigned according to the following abbreviations: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, br s = broad singlet, br t = broad triplet. Purifications were performed by flash chromatography using silica gel (particle size 40–63 μm, Merck) on IsoleraTM (Biotage, Uppsala, Sweden) apparatus.

Palladium on carbon, 3,4-bis(benzyloxy)benzaldehyde (2), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (I) provided the final compound 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (I) as racemic mixture.

5-(3′,4′-Bis(benzyloxy)benzylidene)furan-2(5H)-one (3). Under nitrogen atmosphere, DBU (0.28 mL, 1.88 mmol) was added dropwise to a solution of 2 (158 mg, 1.88 mmol) in dry THF (16 mL). The mixture was stirred for 30 min at room temperature. After cooled down to −10 °C tert-butyl(dimethyl)silyl trifluoromethanesulfonate (0.48 mL, 2.07 mmol) and 1 (600 mg, 1.88 mmol) were added dropwise and the mixture was stirred 1 h at −10 °C, then DBU (0.56 mL, 3.76 mmol) was added dropwise. The reaction mixture was stirred overnight at room temperature, then the solvent was removed under vacuum. Ethyl acetate (20 mL), EtOH (5 mL) and saturated solution of sodium bisulfite (5 mL) were added to the crude residue and stirred overnight at 40 °C. The phases were separated and the organic layer was diluted with ethyl acetate (15 mL), treated with 2.9 N HCl (3 × 20 mL), washed with brine (20 mL), dried and concentrated to afford a sticky black oil. The crude product was purified on silica gel (75:25 cyclohexane/ethyl acetate) to afford the title compound as an orange/brown oil (137 mg, 0.36 mmol, 19% yield).

2H NMR (300 MHz, CDCl3) E isomer: δ = 7.53 (d, J = 7.0 Hz, 4H), 7.49 – 7.24 (m, 9H), 6.92 (d, J = 7.53 (m, 1H), 5.91 (s, 1H), 5.22 (s, 2H), 5.20 (s, 2H).

2H NMR (300 MHz, CDCl3) Z isomer: δ = 7.53 (d, J = 7.0 Hz, 4H), 7.49 – 7.24 (m, 9H), 6.92 (d, J = 6.1 Hz, 1H), 6.21 (d, J = 5.3 Hz, 1H), 5.91 (s, 1H), 5.18 (s, 2H), 5.14 (s, 2H).

5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (I). 2 (275 mg, 0.72 mmol) was dissolved in CH3OH (16.50 mL), and 3% Pd/C (40 mg) was added. The reaction mixture was stirred overnight under hydrogen atmosphere at room temperature, then the catalyst was removed by filtration. The filtrate was concentrated in vacuo to afford the desired product as an orange oil (125 mg, 0.60 mmol, 83% yield).

2H NMR (300 MHz, CD3OD) δ = 6.71 – 6.67 (m, 2H), 6.56 (dd, J = 7.9, 2.2 Hz, 1H), 4.77 – 4.66 (m, 1H), 2.87 (dd, J = 14.1, 6.1 Hz, 1H), 2.78 (dd, J = 14.0, 6.1 Hz, 1H), 2.52 – 2.41 (m, 1H), 2.35 (dd, J = 9.4, 4.7 Hz, 1H), 2.29 – 2.17 (m, 1H), 2.03 – 1.88 (m, 1H).

2.3. Subject selection

A total of thirteen volunteers (mean age 25 ± 4 years and BMI 20.6 ± 2.0 kg m−2) were recruited from students and staff of the
2.4. Study design on healthy volunteers

Subjects were instructed to limit the consumption of polyphenols at least 72 h before experimentation and during the trial. A list of foods to be avoided has been provided to the volunteers. The list included fruits and vegetables rich in polyphenols (e.g., berries, red/purple fruits/vegetables), chocolate and some beverages such as coffee, tea, wine and fruit juice. The study consisted of a randomized, double blind, 2-arm repeated measure cross-over design. One group of subjects consumed 2 capsules of Anthocran® per day, while the other group 2 capsules of Anthocran™ Phytosome™ per day. The experiment was 7-day long. Urine samples were collected before starting supplementation (day 1, time 0) and after the last dosage (day 7) at the following time-points: 1, 2, 4, 6, 10, 12, 24 h. After one week of wash-out the groups inverted the treatment. Each subject received a box containing the number of capsules to consume during the experiment. Capsules were provided in a blind condition. Subjects were instructed to swallow two capsules per day, the first one in the morning before breakfast and the second one before dinner with a glass of water. Three volunteers consumed two placebo capsules with the same shape, size, colour, flavour and excipients of the products tested. Urine samples were collected at the same time-points as previously reported and used as control to verify the influence of diet and circadian rhythm on C. albicans activity. Ethyl gallate 10 µM was added as internal standard in the sample used for the MS analysis and all the samples were stored at −80 °C until analysis.

2.5. Sample preparation

An aliquot (1 mL) of each of the 10 subjects’ urine was centrifuged at 10000 × g for 5 min and the supernatant was extracted on the SPE column, working at 1 mL/min. Salts were removed with water and then all the compounds retained were eluted with 1 mL 100% acetonitrile. The fractions collected were dried under vacuum and then solubilized in 100 µL H₂O-CH₃OH-HCOOH (90:10:0.1, v/v/v). For the quantitative analysis, the stock solutions of the standards were prepared in methanol and then diluted in a pool of the pre-treatment urine samples to obtain the final concentrations for each calibration curve. The samples were then added with ethyl gallate 10 µM as internal standard and processed as described.

2.6. Chromatographic conditions

Cranberry components and urine metabolite separation was performed in a reversed-phase Agilent Zorbax SB-C18 column (150 × 2.1 mm, i.d. 3.5 µm, CPS analitica, Milan, Italy), protected by an Agilent Zorbax guard column, kept at 40 °C, by an UltiMate 3000 system (Dionex) equipped with an autosampler kept at 4 °C working at a constant flow rate (200 µL/min). Each sample (10 µL) was injected into the column and both cranberry components and urine metabolites were eluted with an 80 min multistep gradient of phase A H₂O-HCOOH (100:0.1, v/v) and phase B CH₃CN-HCOOH (100:0.1, v/v): 0–45 min, from 10% B to 20% B; 45–65 min, from 20% B to 60% B; 65–66 min, from 60% B to 90% B; 66–70 min, isotropic of 90% B; 70–71 min, from 90% B to 10% B, and then 71–80 min of isocratic 10% B.

2.7. Polyphenol class identification by HPLC-UV analysis

The identification of polyphenol classes was carried out by HPLC-UV analysis on a HPLC Surveyor LC system (Thermo Fisher Scientific, Milan, Italy) equipped with a quaternary pump, UV–VIS detector (PDA) and an autosampler. The scan range was set from 200 nm to 600 nm. A solution of 4 mg/mL of cranberry extract in H₂O/CH₃OH/HCOOH (90/10/0.1% v/v) was used for the analysis.

2.8. Cranberry component profiling and urine metabolite characterization by high resolution mass spectrometry

Each sample (10 µL) was injected into the RP column as previously described: the cranberry extract was analyzed at a concentration of 4 mg/mL in H₂O-CH₃OH-HCOOH (90:10:0.1, v/v), while the urine samples were analyzed after the treatment described in section 2.3. The analyses were performed on a LTQ-Orbitrap XL mass spectrometer using an ESI source. Mass spectra were acquired in positive and in negative ion modes. A list of 20 background ions was adopted as lock mass values for real time mass calibration [25]. The source parameters used for the positive mode are: spray voltage 4 kV, capillary temperature 300 °C, capillary voltage 30 V, tube lens offset 90 V; for the negative ion mode: spray voltage 4 kV, capillary temperature 300 °C, capillary voltage −23 V, tube lens offset −140 V. The instrument was set up to work in a data-dependent scan mode to acquire both full MS and MS/MS spectra. Full MS spectra were acquired in profile mode by the FT analyzer in a scan range of m/z 100–1200, using AGC scan target 5 × 10⁵ and resolution 30,000 FWHM at m/z 400. Tandem mass spectra were acquired by the linear ion trap (LTQ) which was set up to fragment the 3 most intense ions exceeding 1 × 10⁴ counts. Mass acquisition settings were: centroid mode, AGC scan target 5 × 10⁴, precursor ion isolation width of m/z 3, and collision energy (CID) of 35 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition: 2 repeat counts, 20 sec repeat duration, 30 sec of exclusion duration. Moreover, only singly and unassigned charged ions were fragmented. Instrument control and spectra analysis were provided by the software Xcalibur 2.0.7 and Chromleene Xpress 6.80.

2.9. Targeted and untargeted analyses of cranberry components and metabolites in human urine

An in-house database was created for the targeted analysis by adding all the characterized cranberry extract components as well as known cranberry metabolites identified in other studies and cranberry components deriving from other cranberry sources even if not present in the extract under investigation. The identification was carried out on the QualBrowser tool of Xcalibur 2.0.7 by using the accurate mass and the isotopic and fragmentation patterns. The untargeted analysis consisted of searching for all the ions present in the urine samples collected after the cranberry consumption that were not present or present at intensity relative to noise (< 5 × 10³ counts) in the pre-treatment sample. Spectra analyses were carried out on the QualBrowser tool of Xcalibur 2.0.7 by screening the full MS spectra acquired in negative ion mode in mass ranges of m/z 5 with 10 min as acquisition time for each sample. Each ion detected with these filters was exported with the relative MS/MS spectrum, if present. Identification was performed by following two different approaches based on the accurate mass and isotopic and fragmentation patterns. The first approach consists of giving the precursor ion and the MS/MS spectra list as inputs in the Compound Identification tool of CFM-ID [26], using as mass tolerance error 10 ppm for the precursor ion and 0.3 Da for the fragments. CFM-ID performs a search for candidates in available databases (HMDB and KEGG) based on the accurate mass, then generating in-silico MS/MS spectra of all the candidates and then...
comparing the experimental data with those obtained in-silico. The top candidates were ranked (Jaccard Score) according to how closely they matched and returned to a list. The second approach was initially focused on the calculation of the elemental composition performed on the Elemental Composition page of Xcalibur 2.0.7 by using the following parameters: mass tolerance 10 ppm, charge −1, C, H, O, N, P, S as elements in use. The top 5 formulae were searched in available databases such as PubChem, METLIN, MassBank and in the literature in order to obtain a list of candidates. Following this, the Peak Assignment tool of CFM-ID was used to predict the MS/MS spectra of the putative identified compounds and to compare the in-silico spectra obtained with the experimental spectra.

2.10. Quantitative analysis

The calibration curves for each available metabolite were built by plotting the peak area ratios of metabolite/ethyl gallate versus the nominal concentrations of the metabolite by weighted (1/x2) least-squares linear regression. Table 1 shows all the obtained linear curves and the relative limit of quantification (LOQ). All the samples and calibration solutions were analyzed in triplicates. The areas under the curve of the extracted ion chromatogram of each identified metabolite was integrated by using the Genesis peak algorithm of the Qual Browser tool of Xcalibur 2.0.7.

2.11. Candida albicans biofilm formation assay

The biofilm-forming ability of C. albicans under various conditions (i.e. medium supplementation with cranberry extract or urine fractions) was evaluated on polystyrene 96-well plates using the reference strain C. albicans SC5314 [27]. Prior to experiments, C. albicans was grown overnight in yeast extract, peptone, dextrose (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) broth (YPD) at 30 °C in an orbital shaker. Cells were then harvested and washed with cold phosphate-buffered saline (PBS) and counted by hemocytometer. A standard inoculum of 5 × 10^5 yeast cells/mL was prepared in Roswell Park Memorial Institute 1640 medium (RPMI) and incubated in presence and absence of urine fractions as well as in the presence of Anthocran™.

3. Results and discussion

3.1. Compliance

Volunteers confirmed the consumption of the capsules and the compliance was also verified by counting the capsules in the returned boxes. All the participants had 100% compliance and declared no adverse effects following the intervention.

3.2. Characterization of Anthocran® components

Polyphenol classes of the cranberry extract were characterized by HPLC-UV analysis at typical wavelength: 310–320 nm for phenolic acids, 350–370 nm for flavonoids, 520 nm for anthocyanins, 278 nm for benzoic acids, flavonols and PAs. The identification of each compound in the extract was then obtained on the basis of the accurate mass and of the isotopic and the fragmentation patterns, by acquiring the mass spectra in positive and in negative ion mode. All identified compounds are reported in Table 2, Tables 3 and 4.

3.3. Targeted analysis of human urine after cranberry extract intake

The targeted analysis consisted of searching in the urine samples for the compounds listed in an in-house database (total number of compounds = 138), which comprises the cranberry extract components characterized as reported in section 3.2 and cranberry compounds and metabolites as reported elsewhere [15–21,29,30]. Identification was

| Metabolite                        | [M−H]−  | Slope  | Intercept | R²  | Limit of Quantification (µM) |
|-----------------------------------|---------|--------|-----------|-----|-------------------------------|
| Protocatechuic acid               | 153.0189| 0.1529 | −0.04789  | 0.991| 0.25                          |
| p-Coumaric acid                   | 163.0408| 0.2786 | −0.03032  | 0.995| 0.01                          |
| Gallic acid                       | 169.0146| 0.0253 | −0.00585  | 0.991| 1                             |
| Sinapinic acid                    | 223.0608| 0.0452 | −0.00523  | 0.990| 0.005                         |
| Kaempferol                        | 285.0407| 0.1060 | 0.07999   | 0.991| 0.001                         |
| Quercetin                         | 301.0353| 1.1520 | −0.05015  | 0.999| 0.005                         |
| Syringetin                        | 345.0618| 0.6626 | 0.00924   | 0.981| 0.005                         |
| Quercetin-3-O-arabinofuranoside   | 447.0920| 0.9010 | 0.005247  | 0.997| 0.0025                        |
| Quercetin-3-O-rhamnoside          | 463.0884| 0.5151 | −0.02053  | 0.986| 0.0025                        |
| Quercetin-3-O-galactoside         | 463.0884| 0.5151 | −0.02053  | 0.986| 0.0025                        |
| 2,4-dihydroxybenzoic acid         | 137.0243| 1.1470 | −0.13480  | 0.998| 0.25                          |
| 3,4-dihydroxybenzoic acid         | 137.0243| 0.1113 | −0.01402  | 0.996| 0.05                          |
| 3,4-dihydroxybenzoic acid         | 137.0243| 0.002  | −0.00544  | 0.999| 1                             |
| 2,3-dihydroxybenzoic acid         | 153.0201| 1.2380 | −1.98900  | 0.984| 1.5                           |
| 2,5-dihydroxybenzoic acid         | 153.0194| 0.2438 | 0.67780   | 0.998| 5                             |
| 2,4-dihydroxybenzoic acid         | 153.0175| 0.3176 | −0.33540  | 0.989| 1.5                           |
| 3-(4-hydroxyphenyl)-propionic acid| 165.0561| 0.0048 | 0.00106   | 0.999| 0.25                          |
| 3,4-dihydroxyphenylacetic acid    | 167.0349| 0.0228 | 0.005466  | 0.981| 1.5                           |
| Hippuric acid                     | 178.0598| 0.3032 | 120.80000 | 0.993| 1                             |
| 3,4-dihydroxyhydrocinnamic acid   | 181.0506| 0.2474 | 0.37710   | 0.997| 0.25                          |
| p-Hydroxyhippuric acid            | 194.0450| 0.2756 | 10.42000  | 0.995| 1                             |
| m-Hydroxyhippuric acid            | 194.0446| 0.3605 | −2.94400  | 0.991| 1                             |
| o-Hydroxyhippuric acid            | 194.0461| 0.6801 | −4.23000  | 0.996| 1                             |
| 2-methylhippuric acid             | 192.0670| 0.7954 | 3.605000  | 0.999| 1                             |
| Quinic acid                       | 191.0554| 0.0102 | 0.0097    | 0.999| 0.05                          |
| 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone| 207.0666| 0.2411 | −0.47450  | 0.999| 0.1                           |
performed by considering the following parameters: accurate mass, isotopic pattern, MS/MS fragments and the retention time. In more details for each of the compound listed in the database, the SIC was reconstituted setting as filter ion the m/z calculated for the target compound; when a peak with the same RT of the targeted compound was found, the m/z of the parent ion, the isotopic pattern and MS/MS data were retrieved and compared with that of the standard. For some cranberry components (gallic acid, kaempferol, quercetin, syringetin, quercetin-3-O-arabinoferanoside, quercetin-3-O-rhamnoside and quercetin-3-O-galactoside) the intensity of the parent compound was not intense enough to perform CID experiments and their identity was confirmed by comparing the RT and isotopic pattern with those of genuine standards.

Tables 5 and 6 report the compounds and metabolites identified by using such an approach and setting the ion source in negative ion mode. Specifically, the identification of metabolites reported in Table 5 was confirmed by pure standards, while metabolites listed in Table 6 were putatively identified on the base of the accurate mass, isotopic pattern and, when present, fragmentation pattern. Analyses were also carried out in positive ion mode but no additional components were detected, nor were anthocyanins, which are characterized by an high response in such a polarity mode. The lack of detection of anthocyanins can be explained by considering their biotransformation mediated by the colonic microflora into small phenolic compounds, such as protocatechuic acid, chlorogenic acid, caffeoyl glucose and chlorogenic acid (the most representative flavonol) and related glycoside in the intestine shown that the main metabolites are represented by hydroxyphenylacetic acid catabolites [36,37]. In fact, 3,4-dihydroxyphenylacetic acid was found in urine as possible metabolite of flavonols deriving from intestinal microbiota metabolism.

Proanthocyanidins present in the extract (procyanidins A-type and B-type) were not detected in urine in the present study. Results from previous studies on this class of polyphenols are quite controversial, in particular concerning procyanidin A2, to which several studies attributed the activity of cranberry products in UTI prevention. In most cases [15–19], as in the present study, PACs were not detected in human urine while two works reported PACs in human urine: one work was performed on men and postmenopausal women of 50–70 years who took a single dose of 237 mL of cranberry juice (PACs content in the juice was not reported) [20]; in the second study, performed by the same research group, five young women (20–30 years) consumed 237 mL/day of cranberry juice (140 mg of PACs) according to a weekly schedule for 7 weeks [21]. The results that they obtained showed very low levels of PAC-A2 quantified in human urine (CMAX = 24 ng/mg creatinine) and they concluded that PAC-A2 cannot be used as bio-marker of cranberry intake since there was no correlation with the amount of juice consumed. Taking into consideration all these results, it is commonly accepted that PACs have a very low bioavailability, which decreases as the degree of their polymerization increases [38]. Moreover, it is reported that human microbiota degrades PACs in the colon into phenolic compounds: phenylacetic acids and phenylpropionic acids as metabolites of procyanidins A2, B2, catechin and epicatechin; for procyanidin B2, catechin and epicatechin, valerolactones and valeric acids derivatives have also been reported [39,40]. Although in the present paper the valerolactones origin has not been investigated, we can suggest they come from a microbiota-based transformation of cranberry catechin/epicatechin/PACs. This assumption is supported by independent researchers showing the catechin/epicatechin/PACs bio-transformation to valerolactones is driven by gut microbiota. In particular, a metabolome study based on 2-[14C] (−)-epicatechin in humans showed valerolactones as epicatechin metabolites [41]. Moreover, the bacteria Eggerthella lenta and Flavonifractor plautii were identified as responsible for catechin/epicatechin degradation to valerolactones derivatives by M. Kutscher et al. [42]. Li et al. described phenyl-valerolactones as the main tea catechin metabolites produced by gut microorganisms and detected in human urine and blood [43]. A gamma valerolactone was identified by Appeldoorn M.M. et al. as a main metabolite of procyanidin dimer metabolized by human microbiota [44]. Valerolactones were also detected in human urine by Ottaviani et al. after the consumption of flavanols and PACs [45].

In the present study, the following metabolites deriving from phenylpropionic acids [46] were detected: 3,4-dihydroxyphenylacetic acid, 3-(4-hydroxyphenyl)-propionic acid, dihydroxybenzoic acids,
| Name                                | RT (min) | [M + H]+ Fragments       | [M−H]− Fragments       |
|-------------------------------------|----------|--------------------------|------------------------|
| Flavonols, Flavanols, PACs          |          |                          |                        |
| Coumarin                            | 14       | 147.0446 119 + 91        | 145.0289 –             |
| Scopoletin                          | 17.8     | 193.0501 165 + 152 + 131 + 119 + 105 | 191.0444 –             |
| Kaempferol                          | 55       | 287.0556 259 + 251 + 241 + 231 + 213 + 165 + 153 + 137 + 121 | 285.0399 –             |
| Epicatechin                         | 5.7      | 291.0869 169 + 165 + 151 + 147 + 139 + 121 | 289.0712 245 + 205 + 179 + 161 + 151 + 137 + 125 + 109 |
| Catechin                            | 9        | 291.0869 169 + 165 + 151 + 147 + 139 + 121 | 289.0712 245 + 205 + 179 + 161 + 151 + 137 + 125 + 109 |
| Quercetin                           | 48.5     | 303.0505 257 + 247 + 229 + 165 + 153 + 149 + 137 + 121 | 301.0348 271 + 207 + 179 + 151 + 121 + 107 |
| Epigallocatechin                    | 4.4      | 307.0818 –             | 305.0661 261 + 221 + 199 + 179 + 137 + 125 |
| Gallocatechin                       | 3.1      | 307.0818 –             | 305.0661 261 + 221 + 199 + 179 + 137 + 125 |
| Isoflavonol-3-O-arabinofuranoside   | 55.8     | 317.0661 299 + 285 + 281 + 274 + 257 + 165 + 153 + 139 | 315.0505 287 + 271 + 259 + 243 + 203 + 163 + 151 |
| Myricetin                           | 32       | 319.0454 290 + 273 + 255 + 245 + 165 + 153 + 137 | 317.0298 255 + 227 + 193 + 179 + 151 + 137 + 125 |
| Quercetin-3-O-arabinofuranoside     | 25.2     | 333.0610 301 + 289 + 281 + 277 + 273 + 257 + 165 + 153 + 139 | 331.0454 287 + 271 + 263 + 179 + 151 |
| Quercetin-3-O-arabinopyranoside     | 28.4     | 343.0927 –             | 433.0771 301 + 165 + 153 + 139 |
| Quercetin-3-O-xylopyranoside        | 26.6     | 343.0927 –             | 433.0771 301 + 165 + 153 + 139 |
| Catechin-3-O-gallate                | 16.6     | 343.0927 291 + 273 + 251 + 165 + 153 + 139 | 441.0822 315 + 297 + 289 + 161 + 139 |
| Epicatechin-3-O-gallate             | 20       | 343.0927 291 + 273 + 251 + 165 + 153 + 139 | 441.0822 330 + 305 + 289 + 161 + 139 |
| Kaempferol-7-O-galactoside          | 27.2     | 449.1084 –             | 447.0927 284 |
| Isoflavonol-3-O-arabinofuranoside   | 37       | 449.1084 317             | 447.0927 317 |
| Isoflavonol-3-O-arabinopyranoside   | 38.5     | 449.1084 317             | 447.0927 317 |
| Quercetin-3-O-rhamnoside            | 30.5     | 449.1084 317             | 447.0927 317 |
| Myricetin-3-O-arabinofuranoside     | 16.6     | 451.0877 319             | 449.0720 317 |
| Myricetin-3-O-arabinopyranoside     | 19.6     | 451.0877 319             | 449.0720 317 |
| Myricetin-3-O-xylpyranoside         | 19       | 451.0877 319             | 449.0720 317 |
| Quercetin-3-O-galactoside           | 22.4     | 463.1033 301             | 463.0877 301 |
| Quercetin-3-O-galactoside           | 21.5     | 463.1033 301             | 463.0877 301 |
| Myricetin-3-O-arabinofuranoside     | 34.5     | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-arabinopyranoside     | 32.8     | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-glucosidase           | 31.1     | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-arabinofuranoside     | 38.8     | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-arabinopyranoside     | 39       | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-glucosidase           | 43.5     | 479.1189 317             | 477.1033 315 |
| Myricetin-3-O-glucosidase           | 15.1     | 481.0982 319             | 479.0826 317 |
| Myricetin-3-O-galactoside           | 14.5     | 481.0982 319             | 479.0826 317 |
| Syringetin-3-O-arabinofuranoside    | 43.8     | 490.1346 317             | 491.1189 –             |
| Proanthocyanidin A-type dimer       | 17.23    | 577.1346 437 + 425 + 397 + 287 | 575.1189 425 + 289 + 287 |
| Proanthocyanidin B-type dimer       | 22.6     | 579.1346 453 + 439 + 427 + 409 + 301 + 291 | 577.1346 425 + 407 + 289 |
| Proanthocyanidin A-type trimer      | 16/25/27.3 | 865.1980 713 + 577 + 425 + 287 | 863.1823 575 + 423 + 289 |
| Proanthocyanidin B-type trimer      | 11.5/14.1/24 | 867.2136 579 + 427 + 409 + 291 | 865.1979 577 + 425 + 407 + 287 |
hydroxybenzoic acid and hydroxyhippuric acids, which presence can be related to PACs metabolism.

Phenolic acids represent the main class of identified polyphenols: protocatechuic acid, p-coumaric acid, gallic acid and sinapinic acid were found to be already present in the extract, but they can also derive from the metabolism of other polyphenols as mentioned above; 3,4-dihydroxyhydrocinnamic acid, dihydroxyhydrocinnamic acid-3-O-glucuronide and 3-(4-hydroxyphenyl)-propionic acid can derive from the metabolism of PACs, chlorogenic acid or anthocyanins [17,39,46], while hydroxybenzoic acid, dihydroxybenzoic acids, hippuric acid and hydroxyhippuric acids can derive from the metabolism of all the other flavonoid components [46]. Catechol and pyrogallol derivatives, such as 4-methylcatechol-O-sulphate and pyrogallol-O-2-sulphate that we identified, can be generated from phenolic acids or anthocyanins [46].

| Table 5 | Cranberry components and metabolites identified using the targeted analysis. |
|---------|---------------------------------------------------------------|
| Metabolite identification | RT (min) | Calculated [M−H]− | Observed [M−H]− | MS/MS fragments | Delta ppm |
| Protocatechuic acid | 4.1 | 153.0188 | 153.0189 | 109 | −0.588 |
| p-Coumaric acid | 14.1 | 163.0395 | 163.0408 | 119 | −8.035 |
| Gallic acid | 2.5 | 169.0137 | 169.0146 | – | −5.325 |
| Sinapinic acid | 19.4 | 223.0607 | 223.0608 | 179 + 164 + 149 | −0.717 |
| Kaempferol | 55 | 285.0399 | 285.0407 | – | −2.877 |
| Quercetin | 48.9 | 301.0348 | 301.0353 | – | −1.395 |
| Syringetin | 55.8 | 345.0610 | 345.0618 | – | −2.289 |
| Quercetin-3-O-arabinofuranoside | 28.5 | 433.0771 | 433.0731 | – | 9.306 |
| Quercetin-3-O-galactoside | 21.6 | 463.0877 | 463.0884 | – | −1.684 |
| 2-hydroxybenzoic acid | 24.2 | 137.0239 | 137.0243 | 93 | −3.065 |
| 3-hydroxybenzoic acid | 8.9 | 137.0239 | 137.0231 | – | 5.984 |
| 4-hydroxybenzoic acid | 6.6 | 137.0239 | 137.0232 | 93 | 4.671 |
| 2,3-dihydroxybenzoic acid | 9.1 | 153.0187 | 153.0201 | 109 | −8.561 |
| 2,5-dihydroxybenzoic acid | 6.8 | 153.0187 | 153.0194 | 109 | −3.856 |
| 2,4-dihydroxybenzoic acid | 8.6 | 153.0187 | 153.0175 | 109 | 8.169 |
| 3-(4-hydroxyphenyl)-propionic acid | 12.1 | 165.0552 | 165.0561 | – | −5.816 |
| 3,4-dihydroxyphenylacetic acid | 4.5 | 167.0344 | 167.0349 | – | −2.574 |
| Hippuric acid | 8.2 | 178.0504 | 178.0509 | 134 | −2.640 |
| 3,4-dihydroxyhydrocinnamic acid | 7.1 | 181.0499 | 181.0506 | 137 + 121 | −3.811 |
| p-Hydroxyhippuric acid | 3.6 | 194.0456 | 194.0450 | 150 + 100 + 93 | 3.401 |
| m-Hydroxyhippuric acid | 4.5 | 194.0456 | 194.0446 | 150 + 100 + 93 | 5.256 |
| o-Hydroxyhippuric acid (salicyluric acid) | 14.1 | 194.0456 | 194.0461 | 150 + 100 + 93 | −2.628 |
| 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone | 12 | 207.0657 | 207.0659 | 169 + 122 + 109 | −0.821 |

Table 6 | Metabolites putatively identified using the targeted analysis. |
|-----------------------------------------------|-----------------|-------------------|--------------------|-----------------|-----------------|
| Metabolite putative identification | RT (min) | Calculated [M−H]− | Observed [M−H]− | MS/MS fragments | Delta ppm |
| Isorhamnetin-3-O-arabinopyranoside | 41 | 447.0927 | 447.0954 | – | −5.882 |
| 4-methylcatechol-O-sulphate | 9.7 | 203.0014 | 203.0021 | 123 | −3.300 |
| Pyrogallol-O-2-sulphate | 3.9 | 204.9807 | 204.9814 | 125 | −3.269 |
| Vanillic acid-4-O-sulphate | 4.2 | 246.9912 | 246.9918 | 167 | −2.105 |
| 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-4′-O-sulphate | 9.5 | 287.0225 | 287.0236 | 207 | −3.658 |
| Dihydroxyhydrocinnamic acid-3-O-glucuronide | 9.5 | 357.0821 | 357.0827 | 181 + 137 | −1.484 |

Fig. 2. Searching ions in untargeted analysis – Examples of ions not present in urine before cranberry intake (upper panels), but present in urine samples after the treatment (lower panels): ions at m/z 223.0606 and m/z 207.0665 are present only after the treatment and identified as sinapinic acid (A) and as 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone (B), respectively.
Fig. 3. Compounds identification in untargeted analysis – Single ion chromatograms relative to sinapinic acid and 5′-(3′,4′-hydroxyphenyl)-γ-valerolactone (D) in urine sample before cranberry intake (panels A and D, respectively) and after 10 h and 12 h from cranberry intake (panels B and E, respectively). Peaks with the same RT of genuine standards are only detected after cranberry intake. Final confirmation of identities was achieved by tandem MS analyses. MS/MS spectra of sinapinic acid (C) and 5′-(3′,4′-hydroxyphenyl)-γ-valerolactone (F) with the predicted structure for each fragment assigned by the Compound Identification tool of CFM-ID.

Table 7
Metabolites identified using the untargeted analysis. *identity confirmed by pure standard.

| Metabolite putative identification | RT (min) | Calculated [M−H]− | Observed [M−H]− | MS/MS fragments | Delta ppm | Database |
|-----------------------------------|---------|------------------|----------------|-----------------|-----------|----------|
| 3,4-dihydroxyhydrocinnamic acid*  | 7.1     | 181.0499         | 181.0506       | 137 + 121       | −3.811    | HMDB     |
| Quinic acid*                      | 1.72    | 191.0556         | 191.0554       | 173 + 129       | 1.047     | HMDB     |
| 2-methylhippuric acid*            | 11.1    | 192.0661         | 192.0670       | 74              | −4.894    | HMDB     |
| m-Hydroxyhippuric acid*           | 3.6     | 194.0456         | 194.0450       | 150 + 100 + 93  | 3.401     | HMDB     |
| o-Hydroxyhippuric acid (salicylic acid)* | 4.5 | 194.0456         | 194.0446       | 150 + 100 + 93  | 5.256     | HMDB     |
| Sinapinic acid*                   | 19.4    | 223.0607         | 223.0608       | 179 + 164 + 149 | −0.717    | HMDB     |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone* | 12.0 | 207.0657         | 207.0659       | 163 + 122 + 109 | −0.821    | HMDB     |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone-3′-O-sulphate | 9.5 | 287.0225         | 287.0236       | 207             | −3.658    | HMDB     |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone-4′-O-sulphate | 10.2 | 287.0225         | 287.0233       | 207             | −2.474    | HMDB     |
| 5′-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone-3′-O-sulphate | 6.6 | 303.0175         | 303.0167       | 223             | 2.442     | HMDB     |
| 4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulphate | 6.4 | 305.0331         | 305.0341       | 225             | −3.213    | HMDB     |
| Dihydroxyhydrocinnamic acid-3-O-glucuronide | 9.5 | 357.0821         | 357.0826       | 181 + 137       | −1.484    | HMDB     |
| Salicylic glucuronide             | 9.2     | 370.0774         | 370.0783       | 194 + 150       | −2.513    | PubChem  |
| 3-O-Methylcatechin-sulphate       | 12.3    | 383.0442         | 383.0442       | 303 + 285 + 259 + 244 + 217 + 137 | −1.279    | PubChem  |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone-3′-O-glucuronide | 8.1 | 383.0978         | 383.0981       | 207             | −0.783    | HMDB     |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone-4′-O-glucuronide | 9.4 | 383.0978         | 383.0982       | 207             | −1.044    | HMDB     |
| Sinapinic glucuronide             | 6.8     | 399.0928         | 399.0931       | 223             | −0.877    | HMDB     |
| 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone sulphoglucuronide | 4.6 | 463.0546         | 463.0548       | 383/287/207      | −0.302    | [47]     |
3.4. Untargeted analysis of human urine after cranberry extract intake

As described in the method session, the untargeted approach consists of searching for all the ions present in the urine samples collected after cranberry consumption that were not present or present at intensity relative to noise less than $5 \times 10^2$ counts in the pre-treatment sample. The analysis was performed using the negative ion mode because all the compounds identified using the targeted analysis were mainly detected in this polarity mode. The unidentified compounds, or those recognized as coming from the human basal metabolism (e.g., amino acids), were not included on the list. The search of ions was performed by screening the full MS spectra acquired in negative ion mode using a mass range of $m/z$ 5 and with 10 min as acquisition time for each sample. As an example, Fig. 2A reports the MS spectra resulting by setting a MS range between $m/z$ 220 and 225 and considering a time window between 0 and 10 min and relative to urine collected before (upper panel) and after 10 h (lower panel) the cranberry intake; Fig. 2B reports the MS spectra resulting by setting a MS range between $m/z$ 205 and 210 and considering a time window between 10 and 20 min and relative to urine collected before (upper panel) and after 12 h (lower panel) the cranberry intake. The ions at $m/z$ 223.0606 and 207.0665 are well evident only in the urine samples collected after the cranberry administration but not before. Identification of the unknown compounds was carried out by setting the precursor ion and the MS/MS fragment ions as inputs in the Compound Identification tool of CFM-ID. Fig. 3C and Fig. 3F reports the experimental MS/MS spectra used as input for the Compound Identification tool of CFM-ID which gave sinapinic acid and 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone as best matched results. Final attribution was obtained by comparing RT, MS isotopic pattern and MS/MS fragmentation with those of pure standards (when commercially available). Fig. 3 shows the SIC chromatograms of sinapinic acid (Fig. 3B) and 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone (Fig. 3E) in the urine fraction in which they reached their maximum concentration (10 h for sinapinic acid and 12 h for 5′-(3′,4′-dihydroxyphenyl)-γ-valerolactone), while in the control sample (Figs. 3A and 3D, respectively) they were not present.

Several metabolites were identified in the untargeted analysis as reported in Table 7. Some of them had already been identified using the...
Table 8 summarizes the overall cranberry components and metabolites, including PACs and their metabolites, so far identified in human studies, in comparison with those reported in the present study. Information on the treatment (dose and PACs content of the given cranberry) are also summarized. As already discussed above, PACs were detected only in two studies [20,21] and in one of these [20] the amount of PACs in the cranberry juice was not reported. In these two studies, phenolic acids were reported as PACs metabolites and no valerolactone derivatives were detected as in the study performed by Valentova et al. [15]. Feliciano et al. [17,18] identified several PAC metabolites by using standards, and among these only one valerolactone derivative was identified, while Peron et al. [19] reported a lower amount of metabolites but a higher number of valerolactone/valeric acid derivatives, one of which was also detected in this study. Hence, the untargeted approach here reported has permitted the identification of six valerolactones/valeric acid whose presence in urine after cranberry consumption has never been described before.

3.5. Ex-vivo inhibition of Candida albicans biofilm-formation by urine fractions

Anthocran* (0.1 mg/mL), urine collected before administration of Anthocran®, Anthocran™ Phytosome™ or placebo and urine fractions collected after 1 h, 2 h, 4 h, 6 h, 10 h, 12 h and 24 h of each treatment were tested to investigate their potential ability to reduce C. albicans adhesion and biofilm formation on polystyrene 96-well plates. Anthocran® 0.1 mg/mL was able to strongly reduce the adhesion and biofilm formation (p < 0.0001) of the biofilm-producing strain SC5314 (data not shown). Results expressed as mean ± SD of urine fractions are reported in Fig. 5. Urine samples before (U-Pre) each treatment were inactive, while among the seven fractions tested those collected after 12 h the Anthocran™ consumption (Fig. 5A) as well as Anthocran™ Phytosome™ (Fig. 5B) were shown to significantly inhibit the adhesion compared with the control (p < 0.05 and p < 0.01, respectively for Anthocran* and Anthocran™ Phytosome*). Urine fractions after placebo intake showed no activity at all, meaning that diet and the circadian rhythm does not influence activity. It should be noted that the effect of Anthocran™ Phytosome® at 12 hr superimposes that of Anthocran*, despite the dose of Anthocran* per capsule being 1/3 in the phytosomal preparation (12 mg PACs/capsule Anthocran™ Phytosome™ vs 36 mg PACs/capsule Anthocran®). Phytosomes are lecithin formulations demonstrated to enhance botanical ingredients oral bioavailability both at preclinical and clinical levels [48-51]. The similar ability to reduce C. albicans adhesion and biofilm despite the reduced cranberry extract dose can be consequently explained by considering an increased absorption of active principles allowed by the Phytosome technology. This figure can positively contribute to a more rational and convenient modulation of clinical dosage and posology.
3.6. Metabolite concentration in urine samples

In order to identify the cranberry components/metabolites characteristic of the bioactive fractions and hence most likely responsible for the ability to reduce \( C. \) \( \text{albicans} \) adhesion and biofilm formation, the concentration of the available metabolites for each urinary fraction was obtained through the quantitative analysis explained in the method section. Concentrations of the urinary components are reported as \( \mu \text{M} \) in order to allow the reassemble of the mixtures by using pure compounds and confirm their activities (see below). As examples, Fig. 6 shows two compounds which reached their maximum abundance in the active fraction and two compounds that reached this value in other

Fig. 5. Inhibition of \( \text{Candida albicans} \) biofilm formation – Biofilm biomass was assessed using the crystal violet assay after \( C. \) \( \text{albicans} \) SC5314 strain was cultured for 24 h in RPMI 1640 with/without treatments. A) Activity of urine before Anthocran\textsuperscript{®} intake (U-Pre) and urinary fractions after treatment (U-1 h – U-24 h). Biomass of untreated \( C. \) \( \text{albicans} \) biofilm was used as control (\( C. \) \( \text{albicans} \)). B) Activity of urine fractions (U-1 h – U-24 h) after oral intake of Anthocran\textsuperscript{™} Phytosome\textsuperscript{™} and of urine before Anthocran\textsuperscript{™} Phytosome\textsuperscript{™} intake (U-Pre). Untreated \( C. \) \( \text{albicans} \) biofilm (\( C. \) \( \text{albicans} \)) was used as control. C) Activity of urine fractions (U-1 h – U-24 h) after oral intake of placebo (U-1 h – U-24 h). Control as described for B). Values represent the mean of three independent experiments, and of at least three sample replicates. Significant differences are indicated by *\( p < 0.05 \), **\( p < 0.01 \), ANOVA test.

Fig. 6. Urinary profile of some metabolites – Excretion profiles of 5-(3',4'-dihydroxyphenyl)-\( \gamma \)-valerolactone (A), quercetin (B), \( p \)-coumaric acid (C) and 3,4-dihydroxyphenylacetic acid (D).
Table 9
Cranberry components and metabolites mean (± SD) concentration (µM) in urine fractions. N.D.: not detected.

| Name                              | Pre-treatment | 1 h       | 2 h       | 4 h       | 6 h       | 10 h      | 12 h      | 24 h      |
|----------------------------------|---------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Protocatechuic acid              | 1.22 ± 1.00   | 7.24 ± 3.31 | 4.97 ± 1.52 | 10.81 ± 4.45 | 2.12 ± 1.33 | 3.78 ± 1.47 | 2.03 ± 0.82 | 2.51 ± 0.94 |
| p-Coumaric acid                  | 0.29 ± 0.24   | 13.11 ± 8.30 | 16.63 ± 10.27 | 17.81 ± 8.43 | 3.13 ± 1.62 | 5.40 ± 1.43 | 1.60 ± 0.31 | 0.55 ± 0.28 |
| Gallic acid                      | <LOQ          | 2.28 ± 1.34 | <LOQ      | 1.45 ± 1.06 | <LOQ      | 3.02 ± 1.56 | 2.18 ± 0.91 | 1.22 ± 0.53 |
| Sinapinic acid                   | 0.29 ± 0.19   | 47.02 ± 46.73 | 25.36 ± 24.52 | 68.25 ± 67.90 | 118.75 ± 117.77 | 255.90 ± 247.79 | 84.29 ± 80.26 | 111.53 ± 109.32 |
| Kaempferol                       | N.D.          | 0.083 ± 0.03 | 0.04 ± 0.03 | N.D.      | 0.02 ± 0.01 | 0.04 ± 0.03 | N.D.      | N.D.      |
| Quercetin                        | <LOQ          | 0.108 ± 0.036 | 0.14 ± 0.04 | 0.15 ± 0.01 | 0.15 ± 0.01 | 0.63 ± 0.19 | 0.63 ± 0.27 | 0.18 ± 0.01 |
| Syringetin                       | N.D.          | 0.15 ± 0.14 | 0.02 ± 0.02 | N.D.      | 0.15 ± 0.14 | 0.02 ± 0.01 | 0.01 ± 0.02 | N.D.      |
| Quercetin-3-O-arabinofuranoside  | N.D.          | 0.01 ± 0.01 | 0.03 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.01 ± 0.01 | N.D.      |
| Quercetin 3-O-rhamnoside         | N.D.          | 0.04 ± 0.02 | 0.03 ± 0.01 | 0.02 ± 0.01 | N.D.      | N.D.      | N.D.      | N.D.      |
| Quercetin-3-O-galactoside        | N.D.          | 0.02 ± 0.12 | 0.08 ± 0.06 | N.D.      | N.D.      | 0.01 ± 0.01 | N.D.      | N.D.      |
| 2-hydroxybenzoic acid            | <LOQ          | 0.26 ± 0.11 | <LOQ      | <LOQ      | <LOQ      | 0.85 ± 0.44 | 0.36 ± 0.15 | 0.38 ± 0.18 |
| 3-hydroxybenzoic acid            | N.D.          | 3.86 ± 1.73 | 23.84 ± 16.49 | 18.10 ± 9.90 | 13.91 ± 8.35 | 2.51 ± 1.44 | 7.74 ± 4.65 | 3.71 ± 2.07 |
| 2,3-dihydroxybenzoic acid        | <LOQ          | 2.44 ± 0.53 | 2.20 ± 1.16 | 2.50 ± 0.80 | 1.62 ± 0.69 | 2.99 ± 1.23 | 1.62 ± 0.64 | <LOQ      |
| 2,5-dihydroxybenzoic acid        | <LOQ          | 10.48 ± 4.20 | <LOQ      | <LOQ      | <LOQ      | 14.56 ± 5.40 | 5.97 ± 1.37 | 6.04 ± 2.03 |
| 2,4-dihydroxybenzoic acid        | <LOQ          | 1.51 ± 1.18 | 3.58 ± 2.40 | 4.61 ± 3.44 | <LOQ      | 3.40 ± 3.04 | 1.55 ± 1.22 | 2.88 ± 2.57 |
| 3-(4-hydroxyphenyl)propionic acid| 0.58 ± 0.55   | 25.79 ± 19.77 | 4.22 ± 2.85 | 20.23 ± 7.92 | 17.74 ± 16.76 | 4.10 ± 1.91 | 1.18 ± 0.64 | N.D.      |
| 3,4-dihydroxyphenylactic acid    | N.D.          | 1.49 ± 1.24 | 3.10 ± 2.06 | 9.36 ± 6.36 | 3.85 ± 2.70 | 4.06 ± 2.85 | N.D.      | N.D.      |
| Hippuric acid                    | 66.15 ± 29.07 | 230.12 ± 19.82 | 557.72 ± 26.45 | 409.82 ± 17.03 | 659.46 ± 24.06 | 531.84 ± 68.23 | 192.91 ± 31.13 | 648.52 ± 27.88 |
| 3,4-dihydroxyhydrocinnamic acid  | 0.45 ± 0.40   | 4.39 ± 4.03 | 2.99 ± 2.69 | 3.24 ± 2.43 | 2.50 ± 2.45 | 7.15 ± 4.89 | 4.46 ± 2.05 | 5.08 ± 2.12 |
| p-Hydroxyhippuric acid           | 47.06 ± 21.63 | 1.48 ± 0.49 | 14.14 ± 3.15 | 30.64 ± 4.53 | 59.89 ± 14.55 | 9.04 ± 4.13 | <LOQ      | 46.19 ± 4.34 |
| m-Hydroxyhippuric acid           | 24.88 ± 13.21 | 21.91 ± 3.37 | 37.88 ± 12.61 | 25.77 ± 8.15 | 43.60 ± 10.17 | 32.36 ± 3.54 | 29.03 ± 13.24 | 24.89 ± 3.74 |
| p-Hydroxyhippuric acid           | 36.65 ± 8.21  | 10.84 ± 3.14 | 8.44 ± 3.31 | 7.86 ± 2.68 | 17.99 ± 3.42 | 19.79 ± 1.20 | 13.32 ± 2.78 | 27.48 ± 3.30 |
| 2-methyl hippuric acid           | N.D.          | 2.070 ± 1.11 | 4.85 ± 0.97 | <LOQ      | N.D.      | N.D.      | N.D.      | N.D.      |
| Quinic acid                      | 5.49 ± 3.80   | 12.71 ± 8.02 | 6.42 ± 4.33 | 10.15 ± 8.29 | 9.23 ± 2.26 | 17.36 ± 9.82 | 14.74 ± 7.32 | 8.50 ± 4.92 |
| 5-(3',4'-dihydroxyphenyl)γ-valerolactone | N.D. | 88.34 ± 61.63 | 41.51 ± 15.10 | 26.40 ± 14.03 | 18.33 ± 11.89 | 23.82 ± 12.96 | 245.24 ± 143.59 | 3.83 ± 2.08 |
Fig. 7. Inhibition of Candida albicans biofilm formation by mock mixtures – The activity of the mixtures, prepared using available standards in RPMI medium, representing the inactive fractions (1 h and 6 h) and the active fraction (12 h, *p < 0.05) is reported.

The antiadhesive properties of cranberry, both in vitro and in vivo, have long been reported [52–55] mainly with regard to E. coli, which is the principal uropathogen that causes UTIs. An in vitro study showed that cranberry PACs prevent C. albicans biofilm formation in artificial urine [56]. In the present work, we demonstrate for the first time that cranberry extract as well as some urine fractions, collected after one week of cranberry intake, reduce both C. albicans adhesion and biofilm biomass. Since 12 h urine fractions were the most active, we focused our attention on the components that reached their highest concentration at this time point. The components found to have the highest concentration in this urine fraction are quercetin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and quercetin, respectively, whose TMAX was 12 h; Fig. 6C is relative to p-coumaric acid, with a TMAX reached after 4 h; Fig. 6D shows the excretion profile of 3,4-dihydroxyphenylacetic acid (TMAX = 10 h). Table 9 shows the mean cranberry metabolite concentrations (µM) in the control and in the urine fractions after cranberry intake.

The mixture of the active urine fractions (12 h) and of two inactive fractions (1 h and 6 h) were then reassembled by using available standards dissolved in RPMI and tested on C. albicans. The 12 h mixture was found to significantly reduce adhesion while the other two mixtures were inactive, thus confirming the results of the ex-vivo urine samples (Fig. 7). It is important to underline that the 12 h reconstituted mixture only contained one of the 8 valerolactone derivatives identified (namely, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone) due to the lack of commercial availability of valerolactone standards (their synthesis is on-going in our laboratories). Hence if the bioactivity is related to this class of compounds, as expected, a greater activity could be obtained by integrating the reconstituted mixture with the other seven valerolactones.

The antiadhesive properties of cranberry, both in vitro and in vivo, have long been reported [52–55] mainly with regard to E. coli, which is the principal uropathogen that causes UTIs. An in vitro study showed that cranberry PACs prevent C. albicans biofilm formation in artificial urine [56]. In the present work, we demonstrate for the first time that cranberry extract as well as some urine fractions, collected after one week of cranberry administration, reduce both C. albicans adhesion and biofilm biomass. Since 12 h urine fractions were the most active, we focused our attention on the components that reached their highest concentration at this time point. The components found to have the highest concentration in this urine fraction are quercetin and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. A recent in vitro study [57] demonstrates that valerolactone derivatives display anti-adhesive activity against E. coli, confirming that the in vivo activity is due to PACs metabolites rather than intact PACs. Moreover, quercetin was reported to have in vitro anti-adhesive properties on E. coli [58]. However, the activity could derive not only by a single component but from a synergy of all the metabolites present in that mixture, thus explaining the possible activity of cranberry as a phytocomplex. In fact, many components present in the mixture showed an activity against biofilm formation or anti-adhesive properties against E. coli, like protocatechuic acid, 3, 4-dihydroxyphenylacetic acid and 2-hydroxybenzoic acid [59–61].

In conclusion, the HR-MS method developed allowed the identification of several cranberry components and metabolites in human urine after a highly standardized cranberry extract consumption which has been found to be effective in human studies. PACs were not detected as reported by previous studies, but several metabolites deriving from their catabolism presumably operated by the gut microflora and here not found in vivo were identified. The crude extract and the urine fraction collected at 12 h after cranberry intake were found to be active against C. albicans adhesion ex-vivo. The known metabolite of PACs, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was identified as the most abundant metabolite (245 µM) in the bioactive urine fraction. To our knowledge, this is the first work which demonstrates an ex-vivo inhibition of C. albicans adhesion by human urine after cranberry intake. As a future perspective, as soon as all the identified compounds peaking at 12 h will be available (the synthesis of valerolactones derivatives is on-going) their activity against C. albicans will be evaluated, with a particular interest focused on valerolactone derivatives which represent the most abundant metabolites in urine after cranberry intake. Furthermore, in order to understand whether a synergistic action is involved, compounds will be tested in pure form or in mixture.

A further interesting result of our study derives from the use of the lecithin formulation of the cranberry extract which has shown to markedly increase oral bioavailability and organ target accessibility of cranberry active principles. A clinical study in catheterized subjects is on-going to confirm the effectiveness of Anthocran™ Phytosome™.

Author contributions

GB and GA were the principal responsible for the experimental part and for writing the manuscript. PA, GP, PM AR, MC, GB and GA conceived and designed the study. LF and A. Artasensi contributed to the synthesis of valerolactone derivative. EB and EO contributed to the microbiological studies. CDB and PR contributed to the design of the human volunteers study. GB, AA, LC, LA performed the analyses. All the authors assisted with the manuscript preparation.

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Conflict of interest and sponsorship

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