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Arbutin and its metabolite hydroquinone as the main factors in the antimicrobial effect of strawberry tree (*Arbutus unedo* L.) leaves

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ABSTRACT

Strawberry tree (*Arbutus unedo* L.) leaves are used in folk medicine for treating inflammation, hypertension, and diabetes. The aims of this study were to evaluate the antimicrobial effects of *A. unedo* leaf extracts on 15 uropathogens and test the extent to which arbutin, as the most abundant bioactive compound in the leaves, is responsible for their antimicrobial activity. Antimicrobial activity of leaf extracts against urinary pathogenic microorganisms was tested by agar well diffusion assay and twofolded microdilution method. Since the polyphenolic content responsible for *A. unedo* leaves' antimicrobial activity depends on climate and geolocation, we determined the total phenolic, tannin, flavonoid and phenolic acid content using spectrophotometric methods, arbutin and hydroquinone mass fraction using high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) and antioxidative activities by FRAP, ABTS, and DPPH assay. Although methanol was shown to be a more effective solvent for the extraction of polyphenols from leaves, the traditionally used aqueous extract exhibited similar antimicrobial properties. While arbutin did not show direct antimicrobial activity, its metabolite hydroquinone showed strong antimicrobial activity against the tested uropathogens. The strongest antimicrobial activity of leaf extracts was detected for uropathogenic strains of *Enterococcus faecalis*, which was probably associated with the ability of bacterial β -glucosidase, exerting strong activity in *E. faecalis*, to convert arbutin to hydroquinone. Our study suggested that the aqueous extract of strawberry tree leaves has the potential for use as a phytotherapeutic in clinical application and should be further investigated.

Key words: *Arbutus unedo* leaves; Antioxidative properties; Antimicrobial activity; Arbutin, Hydroquinone, Urinary pathogens.

1. Introduction

Plants produce secondary metabolites like polyphenols that play a vital role in the antioxidant and antimicrobial activity of strawberry tree leaves. The significance of phenolic compounds in their antimicrobial activity has been well-documented and there is a growing interest in plant extracts that could be used as an alternative to current antimicrobial agents with increasing antimicrobial resistance (Cushnie et al., 2003; Ferreira et al., 2012).

The strawberry tree (*Arbutus unedo* L., Ericaceae) is an evergreen shrub that grows in the Mediterranean region, Asia Minor, and Western Europe (Amel, 2013; Malheiro et al., 2012; Mariotto et al., 2008). Its leaves have uroantiseptic, diuretic, and astringent properties and they have been used in folk medicine for treating inflammation, hypertension, and diabetes (Oliveira et al., 2009). A number of ingredients such as tannins, flavonoids, phenolic, and iridoid glucosides have been found in the phenolic fraction of strawberry tree leaves exerting potent antioxidative and antimicrobial activity (Carcache-Blanco et al., 2006; Males et al., 2006; Sanjust et al., 2008).

The phenolic glycoside arbutin was identified as the main bioactive compound in *A. unedo* leaves (Fiorentino et al., 2007; Oliveira et al., 2011). High arbutin content was also present in the *Ericaceae* species *Arctostaphylos uva-ursi* (L.) (bearberry), well known in herbal medicine as an ancient urinary antiseptic and astringent (European Medicines Agency, 2012; Pavlović et al., 2009). Arbutin is absorbed from the gastrointestinal tract where it begins to split up into aglycone hydroquinone and glucose by intestinal microflora under the influence of enzyme β -glucosidase (Blaut et al., 2006). The antimicrobial effect of arbutin is directly dependent on β -glucosidase activity (European Medicines Agency, 2012). Hydroquinone is recognized as an active substance at the site of action (urinary tract) and it might be important for the therapeutic activity of a herbal preparation (Blaut et al., 2006;

Schindler et al., 2002). Since polyphenolic content (including arbutin content) in *A. unedo* leaves depends on climate and geolocation, we investigated the Croatian population of this plant, abundant in the country's coastal area.

To the best of our knowledge, there is no published data on the leaf phenolic acid content or the leaf extract antimicrobial activity for *Klebsiella pneumoniae* ESBL and the clinically isolated microorganisms' strains used in this research. Furthermore, there is no data characterizing the antioxidant and antimicrobial properties of the Croatian population of *A. unedo*. The aims of this study were therefore to determine the content of the major antioxidant compound groups: total phenols, tannins, flavonoids, and phenolic acids and *in vitro* antioxidant and antimicrobial activity of aqueous and methanolic extracts of *A. unedo* leaves. For comparative purposes, the aqueous extract as traditionally used was tested along with methanol, described in the literature as the most effective solvent regarding phenolic extraction from different herbal extracts (Khoddami et al., 2013). In addition, the antimicrobial properties of arbutin as the main compound and hydroquinone as the active metabolite were determined and compared with the antimicrobial properties of *A. unedo* leaf extracts so as to test the extent to which arbutin is responsible for antimicrobial activity.

2. Materials and methods

2.1. Leaf samples

A. unedo leaves were randomly collected in May 2013 on the island of Mali Lošinj (GPS coordinates: 44°31'50" N; 14°28'06" E; 14 m a.s.l.). The collected leaves were dried in a dark place at room temperature. Herbal material was identified by Dr Dario Kremer and deposited in the Fran Kušan Pharmaceutical Botanical Garden at the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. The dried leaves were ground in a laboratory mill and stored in plastic containers at room temperature until analysis.

2.2. Extraction

Powdered dried leaves of *A. unedo* were extracted with water and methanol. The sample (3 grams of powdered leaves mixed with 80 mL of water or methanol) was placed in an ultrasound bath (Bandelin, Sonorex) at 50 °C for 60 min with vortexing every 15 min. Extracts were cooled to room temperature, centrifuged at 4000g for 15 min and filtered through a thick filter paper. Aliquots of water and methanolic extracts were used for determination of total phenolic and tannin content and antioxidant activity. For the determination of antimicrobial properties, water extracts were lyophilized while methanolic extracts were evaporated under reduced pressure at 40 °C (Heidolph Laborota 4000 efficient, HB digital) before lyophilisation. Both *A. unedo* lyophilized extracts were stored in a desiccator due to the plant's hygroscopic characteristics. Yields for water and methanolic extracts were 28.6 and 32.4 %, respectively. To standardize the extract, we determined the total phenolic, tannin, flavonoid and phenolic acid content and the content of arbutin, the most abundant bioactive compound.

2.3. Phytochemical investigations

2.3.1. Total phenolic content

Total phenolic content was determined in water and methanol extracts using the Folin-Ciocalteu spectrophotometric assay (Gao et al., 2002) with slight modifications. Two hundred μL of diluted extract (1:50, v/v) was mixed with 1.35 mL of water and 150 μL of Folin-Ciocalteu reagent. The reaction mixture was incubated at room temperature for 5 min and mixed with 1.5 mL of Na_2CO_3 solution (6 % w/w). Absorbance was measured at 725 nm after 30 min at 50 °C and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry leaf weight.

2.3.2. Total tannin content

Total tannin content was determined by the method of Makkar *et al.* (1993) using the polyvinyl-polyrrolidone (PVPP) to precipitate tannins. Briefly, 30 mg of PVPP was mixed with 2 mL of water, shaken to yield a suspension, and then mixed with 2 mL of diluted leaf extract (1:50, v/v). The solution was well shaken and allowed to stand at 4 °C for 30 min with occasional vigorous shaking. Then, the suspension was centrifuged for 10 min at 3000 rpm and clear supernatants were used for the determination of non-tannin phenolics by the Folin-Ciocalteu method described in Subsection 2.3.1. Tannin content was calculated as the difference between total phenolic and non-tannin phenolic content in the extract.

2.3.3. Total phenolic acids content

The amount of total phenolic acids was determined by spectrophotometric method according to the Rosmarini folium monograph from European Pharmacopoeia (2005). Briefly, 0.200 g of powdered dried leaves was mixed with 80 mL of 50 % ethanol and then extracted on a boiling water bath with a reflux condenser for 30 min. After cooling, the extract was filtered into a volumetric flask and diluted with 50 % ethanol up to 100 mL. One mL of the resulting extract was mixed with 2.0 mL of 0.5 M HCl, 2.0 mL of nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate was dissolved in 100 mL of distilled water), 2.0 mL of 8.5 % sodium hydroxide solution and with distilled water up to 10 mL. A compensatory solution was made by diluting 1.0 mL of extract with distilled water up to 10 mL. Mass fraction (%) of total hydroxycinnamic acid derivatives, expressed as rosmarinic acid, was calculated according to the formula: $A \times 2.5 / m$, where A was absorbance of the test solution at 505 nm, and m was the substance mass in grams.

2.3.4. Total flavonoids content

The amount of total flavonoids was determined by a spectrophotometric method according to Christ and Müller (1960). Powdered dried leaves (0.2 g) were separately extracted for 30 min with 20 mL of acetone, 2 mL of 25 % HCl and 1 mL of 0.5 % solution of

hexamethylenetetramine by heating in a water bath with a reflux condenser until water was brought to the boil. The hydrolysate was passed through cotton wool, and drug residues were extracted again with 20 mL of acetone, heated to boiling for 10 min. This solution was passed through cotton wool again, and the previously described extraction with acetone was repeated three times. The combined filtrates were diluted with acetone to 100 mL. Then, 20 mL of the hydrolysate was mixed with 20 mL of water, extracted with 15 mL of ethyl acetate and after that three times with 10 mL of ethyl acetate. Combined ethyl acetate phases were washed twice with 40 mL of water, then passed through cotton wool and diluted with ethyl acetate to 50 mL. Ten mL of this solution was transferred to two 25 mL flasks. A total of 0.5 mL of 0.5 % aqueous sodium citrate was added to each flask. In one flask, 2 mL of a solution of aluminium chloride (2 g of aluminium chloride hexahydrate dissolved in 100 mL of 5 % methanolic solution of acetic acid) was added. Then, both flasks were supplemented to 25 mL with 5 % methanolic solution of acetic acid. After 45 minutes, yellow solutions were filtered and the absorbance of the developed complex was measured at 425 nm. The blank test was the solution prepared without aluminium chloride. The mass fraction (%) of flavonoids was calculated as quercetin, according to the expression: $A \times 0.772 / b$ (A = absorbance at 425 nm, b = mass of the substance in grams).

2.3.5. HPLC-DAD determination of arbutin and hydroquinone

Mass fractions of arbutin and hydroquinone in leaves were determined by high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) as previously reported (Jurica et al., 2015).

2.4. Antioxidant capacity

2.4.1. Ferric reducing/antioxidant power assay (FRAP assay)

Determination of FRAP antioxidant activity was carried out following the procedure of Benzie and Strain (1996) with slight modifications. The reaction mixture was prepared by

mixing 2.7 mL of FRAP reagent (25 mL of acetate buffer 0.3 M, 2.5 mL of 20 mM FeCl₃ solution and 2.5 mL of 20 mM tripyridyl triazine (TPTZ) solution), 270 µL of distilled water and 150 µL of diluted sample (1:250, v/v) or FeSO₄ working standard and incubated at 37 °C for 40 min. Absorbance was read at 593 nm and the results were expressed as mmol of FeSO₄ equivalents per g of sample.

2.4.2. ABTS^{•+} radical scavenging assay

For determination of trolox equivalent (TE) antioxidant capacity, a method by Re et al. (1999) was applied. Briefly explained, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) stock solution (7 mM in distilled water) and K₂S₂O₈ solution (2.45 mM) were mixed in equal proportions. After 12 hours in the dark to yield the final colour intensity, the solution was diluted until its absorbance, measured at 732 nm, reached 0.700±0.02. The reaction mixture was prepared in a cuvette by mixing 300 µL of the diluted leaf extract (1:250, v/v) and 2.5 mL of ABTS radical solution. The absorbance was measured after 3 min. The difference in initial colouring (A_{0min}) and discoloration after 3 min (A_{3min}) measured at 732 nm was used to calculate the percentage of discoloration. It was plotted against the trolox concentration range and the results were expressed as mg of TE per g of sample.

2.4.3. DPPH[•] radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity of *A. unedo* leaf extracts was evaluated according to the method by Shimada et al. (1992). Five dilution levels (1:500-1:4000, v/v) of the strawberry tree leaf extracts were prepared for each sample using methanol as the dilution solvent. DPPH was dissolved in methanol to produce an initial absorbance between 0.890-0.910. The reaction mixture was prepared by mixing 2 mL of leaf extract and 1.5 mL of DPPH and incubated at room temperature for 30 min. Absorbance was measured at t=0 min and t=30 min. The percentage of absorbance reduction measured at 528 nm was plotted against the concentration of a measured extract and the calibration curve was

constructed for each sample. The results were expressed as the effective concentration (EC₅₀) of the leaf extract, which causes a 50 % decrease of the initial concentration of the DPPH radical.

2.5. Antimicrobial susceptibility testing

2.5.1. Microorganisms and media

The standard laboratory strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ESKL ATCC 700603, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231 and several clinical uropathogenic microorganisms *E. coli* 39636 and 39885; *P. aeruginosa* 40052 and 39868; *K. pneumoniae* 40045 and 39772; *E. faecalis* 39997 and 40080, *C. albicans* 39620, and *C. parapsilosis* were used. All of the microorganisms used in this study were obtained from the culture collection of the Department of Microbiology and Parasitology, Faculty of Medicine, Rijeka, Croatia. The bacteria and yeasts were stored at -80 °C in glycerol broth (10 %) (Biolife, Italy). For the experiments, bacteria were cultured in a Mueller-Hinton broth (MHB) (Difco, MD, USA), and *Candida* in Sabouraud Dextrose Broth (SDB) (Biolife, Italy) for 24 h at 37 °C and 120 rpm (Unimax 1010, Heidolph, Germany). The bacterial and yeast suspensions were adjusted to a concentration of 1.0×10^6 CFU/mL. The 10-fold dilutions of the inocula were cultured on blood agar or Sabouraud Dextrose Agar (SDA) (Biolife, Italy) to verify the absence of contamination and check the validity of the inoculum. The antimicrobial activity of the tested extracts (methanolic and water) and its active compounds (arbutin and hydroquinone) was determined using agar well diffusion and twofolded microdilution methods. Susceptibility tests were made according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2007). The tested extracts and compounds were dissolved in 20 % and 40 % methanol as a

positive control for solvent. Since 20 % methanol has no effect on the microorganisms' sensitivity, it was used as a solvent.

2.5.2. *Agar well diffusion assay*

For the agar-diffusion test, bacterial or yeast suspension was spread onto the surface of Mueller-Hinton agar (MHA) plates (Difco, MD, USA) using sterile swabs. Using a sterile borer, wells of 6 mm in diameter were cut in the agar and filled with 30 μ L of prepared tested solutions. The tested solution was suspended in MHB with concentrations of 512 mg/mL for water and methanolic leaf extracts and arbutin (Sigma-Aldrich, 98 %). The hydroquinone (Sigma-Aldrich, 99 %) concentration was 256 mg/mL. After 2 h of incubation at 4 °C and, in order to achieve better diffusion of the sample in the MHA, an overnight incubation at 37 °C, the plates were examined by measuring the diameter of the growth inhibition zone around the well and the antimicrobial activity was evaluated. The same procedure was used for fungi culture sensitivity with SDA suitable for cultivation of fungi (CLSI, 2008; EUCAST, 2008). The standard antibiotics Norfloxacin, 5 μ g disk (Bio-Rad, USA) (for bacteria) and Fluconazole 25 μ g disk (Becton, Dickinson and Company Sparks, MD, USA) (for fungi) were used as control.

2.5.3. *Twofolded microdilution test*

The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of the tested extracts and active compounds were determined using a standard microdilution technique in MHB for bacterial strains and in RPMI-1640 with 2 % glucose for yeast strains. A series of twofold dilutions with concentrations ranging from 102.4 to 0.1 mg/mL for extracts and for compounds was used in the experiment against every microorganism tested (CLSI, 2012; Jorgensen and Turnidge, 2007; Wiegand et al., 2008). Each sample dilution was mixed with an equal volume of bacterial suspension 1.0×10^5 CFU per well. Positive (broth and inoculum) and negative (simple broth) growth controls were prepared. The plates were

incubated for 24 h, at 37 °C and 120 rpm. The lowest concentrations without visible growth were defined as concentrations that inhibited bacterial or yeast growth (MICs) when compared with the control wells after 24 h of incubation at 37 °C. MBC was measured by inoculating the broths used for MIC determinations onto blood agar and incubated further for 18-24 h. MBC was defined as the lowest concentration of tested samples yielding negative subcultures on the solid medium. As a positive control of growth inhibition, norfloxacin was used in the case of bacteria, fluconazole in the case of fungi. The negative control was water and 20 % methanol. The standard value used as the criterion for the MIC was defined as strong activity for < 0.4 mg/mL, moderate activity for 0.4 – 0.8 mg/mL, and weak activity for > 0.8 mg/mL (Taguri et al., 2006).

2.6. Statistical analysis

All measurements were made in triplicate. Data were expressed as means \pm SD unless otherwise stated.

3. Results

The content of total phenols, tannins, phenolic acids and flavonoids was determined in water and methanolic leaf extracts. A higher amount of total phenols (96.96 mg GAE/g) and tannins (81.29 mg GAE/g) was found in the methanolic extract compared to water extract (66.07 mg GAE/g and 54.48 mg GAE/g for total phenols and tannins, respectively). Total flavonoid and phenolic acid content was 9.9 mg Q/g and 14.8 mg RA/g, respectively. Arbutin content determined by HPLC-DAD was higher in the aqueous extract (3.06 mg/g) than in the methanolic one (2.95 mg/g). Hydroquinone, a pharmacologically active metabolite of arbutin, was not detected in either of the extracts.

The ability of the extracts' to reduce cell oxidative stress was measured by determining antioxidant activity in water and methanolic leaf extracts using FRAP, ABTS,

and DPPH. As summarized in Table 1, the methanolic extract showed better antioxidant power and scavenging radical activity compared to the water extract.

The growth inhibition values of the *A. unedo* leaf extracts, arbutin and hydroquinone for urinary pathogen microorganisms are shown in Table 2. The leaf extracts showed the highest inhibition zone for *C. parapsilosis* 38304 (27 and 28 mm for methanolic and water leaf extracts, respectively). Both the water and methanolic solutions of arbutin showed no antimicrobial activity against the tested microorganisms. The water solution of hydroquinone showed the highest inhibitory value for *E. faecalis* ATCC 29212 and 39997 (27 mm). Methanolic hydroquinone solution had the highest inhibition potential for most of the tested microorganisms compared to the other tested compounds. Clinical strain of *K. pneumoniae* ESBL 40045 was the most sensitive strain with the highest inhibitory zone of 31 mm. The aqueous and methanolic solutions of hydroquinone showed weak or no antifungal activity.

The MIC, MBC, and MFC (minimum fungicidal concentration) for the tested microorganisms are summarized in Table 3. Both extracts of strawberry tree leaves showed the strongest antibacterial activity on clinically isolated strains of *E. faecalis* (MIC < 0.05-0.2 mg/mL). The water and methanolic hydroquinone solutions showed strong antibacterial activity for *E. faecalis* ATCC 29212 and *E. faecalis* 39997 (MIC 0.2 mg/mL) while arbutin solutions showed no antimicrobial activity against the tested microorganisms. Strong antifungal activity for the hydroquinone solutions was obtained for *C. albicans* and *C. parapsilosis* (MIC 0.2 mg/mL).

4. Discussion

Since polyphenolic content seems to be largely responsible for antimicrobial activity, the first goals of this study were to determine the content of total phenols and certain groups of phenolic compounds (tannins, flavonoids, and phenolic acids) in *A. unedo* leaves. The results

of this study showed that methanol was a better solvent for the extraction of phenolics than water as was expected according to data from the literature (Khoddami et al., 2013). The levels of total phenols in this study are similar or slightly higher than the total phenol levels determined in *A. unedo* leaves from Portugal, Turkey and Algeria (Guendouze-Bouchefa et al., 2015; Mendes et al., 2011; Oliveira et al., 2009; Orak et al., 2011), which could be due to sample preparation, climate conditions or time of sample collection (Malheiro et al., 2012). The higher total phenolic content measured in this study might be explained by the use of ultrasound-assisted extraction, which facilitates the extraction of phenolics by breaking the walls of *A. unedo* leaf cells (Khoddami et al., 2013). The mass fraction of total tannins in total phenols in this study (~83 %) was higher than the mass fraction of total tannins in the total phenols in *A. unedo* leaves from Montenegro (~73 %), but lower than the percentage of tannin in total phenols determined in leaves from Greece (~94 %) (Pavlović et al., 2011). The powerful antioxidant effect of strawberry tree leaves could be, among other things, attributable to the high tannin content since previous studies have shown that tannins possess strong antioxidant activity due to the large number of hydroxyl and galoyl groups (Huang et al., 2010). The total flavonoid amount measured in this study was in accordance with those determined by Males et al. (2006) in *A. unedo* leaves from the Pelješac peninsula, and three times lower than the values determined in *A. unedo* leaves from Algeria (Guendouze-Bouchefa et al., 2015). The importance of some flavonoids (e.g. flavonols myricetin, rutin, quercetin, and quercitrin) lies in the fact that they have great power to capture free radicals, while others (catechins) have the ability to chelate metals, thus preventing the possibility of free radical formation (Huang et al., 2010). Phenolic acids, to the best of our knowledge determined in this study for the first time, exhibit a different ability for free radical scavenging depending on the number and position of the hydroxyl and methoxy groups in the molecule (Huang et al., 2010).

Since arbutin is considered to be the most abundant bioactive compound in *A. unedo* leaves, we quantified this glycoside as well as its pharmacologically active metabolite hydroquinone using HPLC-DAD. The values obtained for the arbutin content in this study are comparable with the results obtained in other studies, which ranged from 0.6 mg/g (Fiorentino et al., 2007) to 12.4 mg/g (Pavlović et al., 2011). Hydroquinone was not detected in the leaves, which is consistent with the results of previously published results (Jurica et al., 2015; Pavlović et al., 2011).

Antioxidant activity determined by FRAP, ABTS and DPPH indicated a better antioxidative effect of the methanolic extract. Guendoze-Bouchefa et al. (2015) determined a stronger radical scavenging effect (ABTS) compared to this study, which can be attributed to three times higher content of flavonoids and phenolics in their methanolic leaf extracts. The ability to scavenge the DPPH radical for water extract was comparable to the values from previous studies (Malheiro et al., 2012; Oliveira et al., 2009) while the methanolic leaf extract showed better activity compared with previous studies (Guendouze-Bouchefa et al., 2015; Oliveira et al., 2009). The methanolic extract was more effective due to the higher tannins and phenolic acids content, which are more likely extracted with methanol than with water.

The second goal of this study was to investigate strawberry tree leaf antimicrobial activity against urinary pathogens which have only partially been investigated till now but have a high potential to develop resistance and mutate in the human body. To the best of our knowledge, this was the first time that the susceptibility of *Klebsiella pneumoniae* ESBL to *A. unedo* leaf extracts was tested. Also, this is the first study to include susceptibility testing of such a large number of microorganisms in relation to the polyphenolic content and antioxidant activity of *A. unedo* leaf extracts. The aqueous extract as traditionally used was tested together with methanol for comparative purposes. Besides *A. unedo* leaf extracts, we studied the antimicrobial properties for the phenolic glycoside arbutin as the main compound present in

leaves and its metabolite hydroquinone to test to what extent arbutin is responsible for antimicrobial activity. First, the inhibition zones for the tested agents (water and methanolic leaf extracts, arbutin and hydroquinone) were measured. After that, MIC and MFC were determined.

Susceptibility testing by agar well diffusion method for *E. faecalis* strains pointed to a weak antibacterial activity for aqueous and methanolic extracts, which was in accordance with studies by other authors (Ferreira et al., 2012; Kivçak et al., 2001). However, in this study the authors have shown a strong antibacterial effect of leaf extracts on clinically isolated strains of *E. faecalis* with considerable MIC values (<0.05-0.2 mg/mL). Similar MIC value (0.112 mg/mL) was determined in *A. unedo* leaf petroleum extracts presented by Dib et al. (2010). These values may be associated with increased extracellular β -glucosidase activity, which is secreted by *E. faecalis*. The antimicrobial effect of arbutin present in leaves strongly depends on the extracellular activity of β -glucosidase, an enzyme responsible for converting arbutin to free hydroquinone, which is 100 % active for the *E. faecalis* strain (European Medicines Agency, 2012). *K. pneumoniae* with the ability to produce extended-spectrum beta-lactamases (ESBL) was tested, as far as we know, for the first time and was not susceptible to leaf extracts. The same results were obtained by other authors for different extracts and different *Klebsiella* strains (Ferreira et al., 2012; Pavlović et al., 2014). Only Dib et al. (2010) pointed to a strong antibacterial effect for *K. pneumoniae* in a petroleum extract (MIC 0.108 mg/mL). Such an effect could be related to monoterpenes isolated from *A. unedo* leaves, which are able to penetrate into the cell or destroy the cell membrane as well as the other compounds present, such as α -pinen and sabinen previously isolated from strawberry tree leaves (Dib et al., 2010; Magiatis et al., 1999; Matasyoh et al., 2007; Oussou et al., 2008; Sikkema et al., 1995). Water and methanolic leaf extract showed a weak inhibition effect for *E. coli* and *P. aeruginosa* which correlated with other surveys for *A. unedo* leaf extracts (Dib et al., 2010; Ferreira et al.,

2012; Guendouze-Bouchefa et al., 2015; Kivçak et al., 2001; Malheiro et al., 2012). This research showed weak inhibitory effect of *A. unedo* leaf extracts for *C. albicans* which is in correlation with other studies (Dib et al., 2010; Ferreira et al., 2012; Kivçak et al., 2001). A clinically isolated strain of *C. parapsilosis* exerted resistance on leaf extracts. Arbutin did not show any antimicrobial activity.

Since arbutin could be transformed into hydroquinone in some bacterial species, the authors also decided to determine MIC and MFC values for hydroquinone. Hydroquinone showed the strongest antimicrobial effect compared with leaf extracts and arbutin. Susceptibility evaluation by agar well diffusion assay for hydroquinone toward clinically isolated fungi *C. albicans* and *C. parapsilosis* showed no significant results, although the MIC values were 0.2 mg/mL, respectively. A similar effect was observed for hydroquinone dissolved in water for *K. pneumoniae* ESBL 39772 (MIC 0.8 mg/mL). The differences in microorganism susceptibility testing by screening method and the determined MIC values are likely to be associated with the possibility of a precipitate formation that interfered with the diffusion of phenolic compounds through the agar (Cushnie et al., 2003).

The different antimicrobial properties of *A. unedo* leaves for the same bacterial strains relate to the various extracting agents used and amount of extracted compounds (El Ouarti et al., 2012; Ferreira et al., 2012; Kivçak et al., 2001; Pavlović et al., 2014). The antimicrobial efficiency of *A. unedo* leaf extracts, other than arbutin itself, could be related to the presence of phenolic acids (cinnamic, ferrulic, caffeic) and their esters, which inhibit bacteria and fungi growth (Huang et al., 2010; Silici et al., 2007). The mode of action of these compounds is associated with irreversible changes in the bacterial cell membranes (cellular charge, intracellular and extracellular permeability, physical and chemical properties) (Borges et al., 2013).

5. Conclusions

In summary, we demonstrated that the content of different phenolic groups in *A. unedo* leaf extracts is comparable to other studies, and the differences could be associated with climate conditions or the sample preparation method. A strong antioxidative and radical scavenging activity was proven for *A. unedo* leaves. Leaf extracts showed the strongest antibacterial effect on clinical strains of *E. faecalis*. Although methanol extracted higher amount of polyphenolics from leaves, the authors found that the aqueous leaf extract that would be used in clinical practice exhibited similar antimicrobial properties. The most responsible component in the treatment of asymptomatic bacteriuria is probably hydroquinone, formed with the assistance of β -glucosidase from arbutin (Blaut et al., 2006). Hydroquinone showed considerable antimicrobial properties and low MIC values for all of the tested urinary pathogens. The absence of β -glucosidase activity in some of the tested microorganisms is probably responsible for the low antimicrobial properties of leaf extracts. This study has confirmed the antimicrobial potential of *A. unedo* leaves in the treatment of asymptomatic urinary infections. The effectiveness for this treatment depends on the amount of phenolic compounds, especially arbutin in leaves. Our results clearly demonstrate that the aqueous extract of *A. unedo* leaf extract could be used as a phytotherapeutic agent; however, such a possibility needs to be investigated through clinical application.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Antioxidative activity of *A. unedo* leaves.

Method	Water extract	Methanolic extract
FRAP* (mmol FeSO ₄ /g)	1.187±0.013	1.896±0.022
ABTS* (mg TE/g)	130.172±3.365	165.510±0.382
DPPH** [EC ₅₀ (mg/mL)]	0.055	0.040

FRAP – ferring reducing antioxidative power; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation; DPPH – 2,2-diphenyl-1-picryl-hydrazyl-hydrate cation. TE – trolox equivalent; EC₅₀ – substrate concentration to produce 50 % reduction of the DPPH; * results expressed on dry leaf mass weight (g); ** results expressed in leaf extract volume (mL).

Table 2. Inhibition zones for the *A. unedo* leaf extracts, arbutin and hydroquinone.

Microorganism	Strain	Water solution			Methanolic solution (MeOH 20 %)			Anti- biotic
		AqE 512 mg/mL	ARB 512 mg/mL	HQ 256 mg/mL	MeE 512 mg/mL	ARB 512 mg/mL	HQ 256 mg/mL	Norflo- xacin 10 µg
Inhibition zone (mm)								
<i>E. coli</i>	ATCC 25922	–	–	13	–	–	16	37.5
	39636*	–	–	17	13	–	20	11
	39885*	–	–	15	–	–	16	29.5
<i>P. aeruginosa</i>	ATCC 27853	10	–	19	22	–	14	37.5
	40052*	10	–	16	14	–	22	29
	39868*	–	–	15	12,4	–	27	29.5
<i>K. pneumoniae</i> <i>ESBL</i>	ATCC 700603	–	–	15	–	–	16	26
	40045*	–	–	14	–	–	31	28.5
	39772*	–	–	–	9	–	17	17
<i>E. faecalis</i>	ATCC 29212	–	–	27	8	–	26	26.5
	39997*	–	–	27	14	–	20	19
	40080*	–	–	21	12	–	25	23
								Fluco- nazole 25 µg
<i>C. albicans</i>	ATCC 10231	–	–	–	–	–	–	28
	39620*	–	–	–	–	–	–	29
<i>C. parapsilosis</i>	38304*	28	–	–	27	–	12	23

AqE – strawberry tree leaf water extract; MeE – strawberry tree leaf methanolic extract; ARB – arbutin; HQ – hydroquinone; (*) – clinically isolated strain; (–) no effect. Results are expressed as mean (n = 3).

Table 3. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of *A. unedo* leaf extracts, arbutin and hydroquinone.

Microorganism	Strain	Water solution						Methanolic solution (MeOH 20 %)									
		AqE			HQ			MeE			ARB			HQ			
		MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	
<i>E. coli</i>	ATCC 25922	25.6 ^C	>51.2	>25.6	0.4 ^B	0.4	0.4	6.4 ^C	>51.2	>25.6	>25.6	0.4 ^B	>25.6	>25.6	>25.6	0.4 ^B	0.4
	39636*	25.6 ^C	>51.2	>25.6	0.4 ^B	0.4	0.4	6.4 ^C	51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
	39885*	25.6 ^C	>51.2	>25.6	0.4 ^B	0.4	0.4	6.4 ^C	51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
<i>P. aeruginosa</i>	ATCC 27853	25.6 ^C	51.2	>25.6	0.4 ^B	0.4	0.4	6.4 ^C	25.6	>25.6	>25.6	0.4 ^B	>25.6	>25.6	0.4 ^B	0.4	
	40052*	25.6 ^C	51.2	>25.6	0.4 ^B	0.4	0.4	1.6 ^C	51.2	>25.6	>25.6	0.4 ^B	>25.6	>25.6	0.4 ^B	0.4	
	39868*	25.6 ^C	51.2	>25.6	0.4 ^B	0.4	0.4	6.4 ^C	51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
<i>K. pneumoniae</i> <i>ESBL</i>	ATCC 700603	12.8 ^C	>51.2	>25.6	0.8 ^B	0.8	0.8	6.4 ^C	>51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
	40045*	12.8 ^C	>51.2	>25.6	0.8 ^B	0.8	0.8	3.2 ^C	>51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
	39772*	25.6 ^C	>51.2	>25.6	0.8 ^B	0.8	0.8	3.2 ^C	>51.2	>25.6	>25.6	0.8 ^B	>25.6	>25.6	0.8 ^B	0.8	
<i>E. faecalis</i>	ATCC 29212	25.6 ^C	25.6	>25.6	0.2 ^A	0.2	0.2	25.6 ^C	25.6	>25.6	>25.6	0.2 ^A	>25.6	>25.6	0.2 ^A	0.2	
	39997*	0.2 ^A	>51.2	>25.6	0.2 ^A	>0.2	>0.2	0.1 ^A	>6.4	>25.6	>25.6	0.2 ^A	>25.6	>25.6	0.2 ^A	0.4	
	40080*	0.1 ^A	25.6	>25.6	0.4 ^B	0.4	0.4	<0.05 ^A	6.4	>25.6	>25.6	0.4 ^B	>25.6	>25.6	0.4 ^B	0.4	
<i>C. albicans</i>	ATCC 10231	51.2 ^C	51.2	25.6 ^C	25.6	25.6	25.6	51.2 ^C	51.2	25.6 ^C	25.6	25.6 ^C	25.6	25.6	0.2 ^A	0.2	
	39620*	51.2 ^C	51.2	25.6 ^C	25.6	25.6	25.6	51.2 ^C	51.2	25.6 ^C	25.6	25.6 ^C	25.6	25.6	0.2 ^A	0.2	
	38304*	51.2 ^C	51.2	25.6 ^C	25.6	25.6	25.6	51.2 ^C	51.2	25.6 ^C	25.6	25.6 ^C	25.6	25.6	0.2 ^A	0.2	

AqE – *A. unedo* leaf water extract ; MeE – *A. unedo* leaf methanolic extract; ARB – arbutin; HQ – hydroquinone; MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; MFC – minimum fungicidal concentration; * clinically isolated strain; A – strong activity; B – moderate activity; C – weak activity. Results are expressed as mean (n = 3).