Antimicrobial activity and phytochemical screening of *Arbutus unedo* L.

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1. Introduction

In the Mediterranean region and North Africa, *Arbutus unedo* L. is traditionally used as an alternative medicine for its biological properties. The fruit has antiseptic, diuretic and laxative effects, and the leaves have astringent, urinary tract antiseptic, anti-diarrheal and depurative properties (Ziyyat et al., 1997; Kivcak and Mert, 2001; Pabucuoglu et al., 2003; Mariotto et al., 2008; Afkir et al., 2008). Ziyyat and Boussairi, 1998 and Ziyyat et al., 2002 showed that an aqueous extract of *A. unedo* exhibited antihypertensive (Haouari et al., 2007) and vasorelaxant properties (Rosato et al., 2001). Furthermore, an *in vitro* study indicated that diethylthylether and ethyl acetate extracts of *A. unedo* leaves have an anti-aggregating effect on human platelets (Redondo et al., 2005a,b). This effect is likely mediated by its antioxidant activity, which may inhibit protein tyrosine phosphorylation and Ca2+ influx into platelets (Redondo et al., 2005a,b; Oliveira et al., 2009). Several compounds have been isolated from *A. unedo*, including aromatic acids, iridoids, monoterpenoids, phenylpropanoids, sterols and triterpenoids (Carcache-Blanco et al.,...
A. unedo. For example, Ayaz et al., 2000 detected lactic, malic, suberic and fumaric acids. Phytochemical studies have shown that the leaf extract contains phenolic antioxidant compounds, such as flavonoids (quercitin, isoqueretin, kaempferol, hyperoside and rutin) (Mazza and Miniti, 1993; Males et al., 2006), tannins, phenolic glycosides, anthocyanins, gallic acid derivatives, (+)-catechin and (+) catechin gallate (Fiorentino et al., 2007). Also several compounds have been isolated from the roots of A. unedo such as (+)-catechin, (+) catechin gallate and a number of phenolic compounds were also identified by GC–MS such as benzeneacetic acid 4-hydroxy, caffeic acid, gallic acid, protocatechic acid and bis(2-ethylhexyl) phthalate (Dib et al., 2010). Therefore, the aim of the present work was: (i) to do a primary phytochemical screening of the main secondary metabolites classes and determined the polyphenols content contained in the root extract, (ii) to examine the antimicrobial activity of water and methanol extract, and three phenolic fractions from A. unedo on selected bacterial pathogens.

2. Experimental

2.1. Plant materials

Samples of A. unedo roots were collected in Terni Forest (about 20 km South of Tlemcen, Algeria; altitude, 1190 m; 34° 49’ N, 1° 19’ E) in September 2008. Voucher specimens were deposited in the herbarium of the Tlemcen University Botanical Laboratory (voucher no. Er 09.08). A portion of each sample was stored at 4 °C for future studies.

2.2. Preparation of the extracts

2.2.1. Methanol extract

Roots were placed into the extractor of a Soxhlet. The extraction was carried out by using solvents of increasing polarity starting from hexane, dichloromethane, and methanol. At the end of the extraction the methanol extract was concentrated by evaporation.

2.2.2. Water extract

The water extract was prepared by cold maceration of 150 g of roots in 500 ml of distilled water for 48 h. Then the extract was filtered, concentrated, and dried in vacuo.

2.3. Phytochemical screening

2.3.1. Qualitative analysis

The phytochemical tests to detect the presence of tannins, flavonoids, anthocyanins, saponins, coumarines, quinones, anthraquinones, reducteurs compounds and alkaloids were performed according to the method described by Kokate, 1994 and Harborne, 1998. The tests were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents. The results for the extracts studied are shown in Table 1.

2.3.2. Quantitative analysis

The total flavonoids, anthocyanins and flavones & flavonols contents were determined spectrophotometrically using the Neu reagent (2-aminoethyl-diphenyl borate). This reagent is based on method Dohou et al., 2003 and Lebreton et al., 1967 that is a colorimetric method. All tests were carried out in triplicate.

2.3.3. Isolation and extraction of polyphenols fractions

100 g of roots were crushed and extracted with 850 ml of water, 360 ml of methanol, then 650 ml of acetone at room temperature during 24 h. After filtration, acetone and methanol were removed in vacuo. The aqueous phase was extracted twice with 150 ml of methylene chloride in order to eliminate pigments and lipids and then lyophilized. The dry powder obtained was extracted four times with 150 ml of ethyl acetate. The combined organic layers were subsequently dried on MgSO₄. Filtration and concentration in vacuo yielded 1.65 g of a red solid (0.55% yield). A part (1.50 g) of crude extract was solubilised in a mixture MeOH/H₂O 7:3 (v/v) and subjected to column chromatography through silica gel (65 g). Elution with 50% methanol/water (v/v) containing 0.1% (v/v) acetic acid gave three fractions Fr-1, Fr-2 and Fr-3 accounting for 10.66%, 39.33%, 25.33% of the whole extract, respectively, all being mixtures (Table 2).

2.4. Antimicrobial assay (disk diffusion assay)

The bacterial strains were used to assess the antimicrobial properties of the test samples, two Gram-negative strains: E. coli (ATCC 25922), P. aeruginosa (ATCC 27853) and one Gram-positive S. aureus (ATCC 25923). Bacterial strains preserved in nutrient agar at 4 °C, were revivified in nutrient solution and incubated at 37 ± 1 °C during 18–24 h. 0.1 ml of each culture was added to 10 ml BHIB (Brain Heart Infusion Broth, Pronadisa Hispanalab). For antimicrobial assay, bacterial strains were grown on Mueller-Hinton Agar (MHA, Pronadisa

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**Table 1** Phytochemicals detected in extracts of roots of A. unedo.

<table>
<thead>
<tr>
<th>Compounds groups</th>
<th>Water extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reducteurs compounds</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Saponins</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Coumarins</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Key: + = present; – = absent

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**Table 2** Chromatographic features of phenolic fractions of roots.

<table>
<thead>
<tr>
<th>Physical aspect</th>
<th>Sample weight (g)</th>
<th>Column chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Compounds</td>
</tr>
<tr>
<td>Red solid</td>
<td>1.5</td>
<td>Fr-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fr-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fr-3</td>
</tr>
</tbody>
</table>
Hispanalab). Bacterial inocula reached microbial densities in the range $10^6$–$10^7$ cfu ml$^{-1}$.

2.5. Antimicrobial activity

Two different techniques were used to test the anti-microbial activity: the paper disc diffusion and the dilution agar method. The minimum inhibitory concentration (MIC) was determined by the later method.

2.5.1. Paper disc diffusion method

Paper discs (6 mm in diameter) saturated with 30 µg ml$^{-1}$ solution of plant extract were applied to the surface of agar plates that were previously seeded by spreading of 0.1 ml overnight culture. The plates were incubated overnight at the appropriate temperature (see above) and the diameter of the resulting zone of inhibition was measured in millimeters. The results indicated in Table 3 and in the text represent the net zone of inhibition including the diameter (6 mm) of the paper disk. The scale of measurement was the following (disk diameter included): >20 mm zone of inhibition is strongly inhibitory; <20–12 mm zone of inhibition is moderately/mildly inhibitory and <12 mm is no inhibitory. All the data collected for each assay are the average of three determinations.

2.5.2. Dilution agar method

A dilution agar method was used to determine the Minimum Inhibitory Concentrations (MIC). Stock solutions were obtained by dissolving extracts in dimethylsulfoxide (DMSO 1%). Serial dilutions were made to obtain concentrations ranging from 0 to 800 µg ml$^{-1}$ for methanol and water extracts and phenolic fractions. Each mixture was added to Mueller–Hinton agar for bacteria (Cowan, 1999; Lennette et al., 1985). The Petri dishes contained a sterile solution of DMSO and the culture medium, respectively, after incubation at 37°C for 24 h. The experiments were performed in triplicate.

3. Results

3.1. Phytochemical screening

With the increase in the incidence of resistance to antibiotics, alternative natural products of plants could be of interest. Some plant extracts and phytochemicals are known to have antimicrobial properties, which could be of great importance in the therapeutic treatments. In the last years, various studies have been conducted in different countries, demonstrating the efficacy of this type of treatment (Coutinho et al., 2008). Table 1 shows the presence of various compounds such as quinones, anthraquinones, reducteurs compounds, anthocyanins, flavonoids and tannins. However alkaloids, saponins and coumarins were not detected. Through phytochemical prospecting of the extracts, it was possible to determine the presence of diverse classes of secondary metabolites that show a wide variety of biological activities such as antimicrobial (Djipa et al., 2000; Esquenazi et al., 2002), antioxidant (Barreiros and David, 2006), antitumor and antiphitic (Okuda et al., 1989). The result of total flavonoids, anthocyanins, flavones and flavonols contents determined spectrophotometrically is shown in Fig. 1. The roots of A. unedo were strongly dominated by anthocyanin compounds (3.65 mg g$^{-1}$) followed by total flavonoids (0.56 mg g$^{-1}$) and flavones & flavonols (0.17 mg g$^{-1}$).

3.2. Antimicrobial activity (assay disk)

Preliminary screening of the antimicrobial activity in vitro of methanol and water extract, and three phenolic fractions of roots was studied against three pathogen microorganisms using the filter paper disc agar diffusion technique. The disc diffusion assay was only used as an indication of anti-microbial activity since the amount of extract or fractions that adhered to the disc was not quantitatively determined. The results showed variation in the antimicrobial properties of plant extracts (Table 3). E. coli was found to be the most inhibited pathogen by the water extract with a diameter of zone of inhibition from 30 mm. The Fr-2 and Fr-3 exerted also potential effect of antibacterial activity against S. aureus with diameters of zones of inhibition from 20 and 22 mm, respectively. The methanol extract was active against S. aureus (15 mm). However, Fr-1 fraction exhibited no effect of antibacterial activity against of the bacterial pathogens tested (Table 3).

3.3. Minimum inhibitory concentrations (MIC)

The antibacterial activities of both extracts and phenolic fractions of A. unedo against the employed bacteria were

| Table 3 Antimicrobial activity of extracts and phenolic fractions from the roots. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Microorganisms      | Inhibition zone (mm) | Fractions | | |
|                    | Water | Methanol | Fr-1 | Fr-2 | Fr-3 |
| E. coli            | 30 | 15 | i | i | i |
| P. aeruginosa      | 9 | i | i | 13 | 16 |
| S. aureus          | 12 | 11 | i | 20 | 22 |
Table 4 Minimal inhibitory concentration (MIC) (µg ml⁻¹) of root extracts and phenolic fractions.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Extracts</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Methanol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>200</td>
<td>600</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>&gt;800</td>
<td>&gt;800</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;800</td>
<td>&gt;800</td>
</tr>
</tbody>
</table>

qualitatively and quantitatively assessed by the presence or absence of inhibition zones.

As shown in Table 4, water and methanol extract of roots are effective against *E. coli*, (minimum inhibitory concentration [MIC] = 200 and 600 µg ml⁻¹). Fr-3 exhibited activity against *S. aureus* and *P. aeruginosa* with an MIC of 200 and 500 µg ml⁻¹, respectively. Fr-2 was active against *S. aureus* with an MIC of 400 µg ml⁻¹. However Fr-1 shows no inhibition toward any of the microorganisms assayed (Table 4).

4. Discussion and conclusion

The water and methanol extract of roots of *A. unedo* evaluated in this work has different varieties of phytochemicals that could be considered as responsible for the antimicrobial activity. Although they usually occur as complex mixtures, their activity can generally be accounted for in terms of their major components. The antimicrobial activity of the phenolic fractions could be due to the investigated strain sensitivity to tannins, flavonoids and the other phenolic components. (Djipa et al., 2000; Esquenazi et al., 2002). The tannin components of epicatechin and catechin demonstrated strong antimicrobial activity against bacteria and fungi (Ho et al., 2001). Flavonoids are synthesized by plants in response to microbial infection (Dixon et al., 1983) and are effective against a broad range of microorganisms. This study confirms that the roots of *A. unedo* contain high amounts of polyphenol compounds. The phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects including antimicrobial activity. In conclusion, poor antibacterial activity against both *S. aureus* and *P. aeruginosa* bacteria was shown with water and methanol extract. However moderate antibacterial activity was shown by water extract and both fractions (Fr-2 and Fr-3) against *E. coli* and *S. aureus*, respectively.

References


