Chemical Composition and Biological Prospects of Essential Oils and Extracts of *Aphyllocladus spartioides* Growing in Northwest Argentina

Liliana Celaya,a,b Carmen Viturro,*a and Luís R. Silva,a,c,d

aPRONO/A Laboratorio de Productos Naturales del NOA, CIT-Jujuy-Universidad Nacional de Jujuy-CONICET, Italo Palanca 10, CP4600 S. S. de Jujuy, Argentina, e-mail: civiturro@arnet.com.ar; lilianacelaya@hotmail.com

bREQUIMTE/Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, 228 Rua de Jorge Viterbo Ferreira, 4050-313 Porto, Portugal

cLEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal

dCICS – UBI – Health Sciences Research Centre, University of Beira Interior, 6201-506 Covilhã, Portugal

*Aphyllocladus spartioides* WEDD. is a native and aromatic herb used in traditional medicine, however it is still poorly investigated. In this work, the volatile profile of *A. spartioides* growing in Hornillos-Northwest Argentina was determined by GC/MS/FID and the phenolic compounds of hydroethanolic and decoction extracts were analyzed by HPLC-DAD. The antibacterial potential, antioxidant activity and α-glucosidase inhibition activity were checked by *in vitro* assays. The volatile profile allowed the identification of 68 compounds, being α-pinene and cadinene the main ones. Eighteen phenolics were identified, isorhamnetin derivatives and different phenolic acid derivatives were found in higher amounts, mainly in the hydroethanolic extract. A concentration-dependent activity was noticed against DPPH, O₂⁻, NO and α-glucosidase, these activities being reported for the first time. Hydroethanolic extract was most active against DPPH, NO and α-glucosidase (IC₅₀ = 79, 206 and 181 μg/ml). Decoction extract proved to be better against O₂⁻ (IC₅₀ = 20 μg/ml). Regarding the antibacterial activity, hydroethanolic extract was more active compared with decoction and essential oil. MICs of 0.3 – 0.6 mg/ml were obtained against *Staphylococcus aureus*, *Bacillus cereus*, and *Micrococcus luteus*. Results suggest that the extracts of *A. spartioides* from Northwest Argentina may be interesting to use as a source of natural antioxidants/antimicrobials for pharmaceutical incorporations or food supplementation.

**Keywords:** *Aphyllocladus spartioides*, Essential oils, Polar extracts, Bioactive compounds.

Introduction

The Puna or Altiplano in Northwest Argentina is an arid environment at high altitude. Plants from these regions constitute a genuine source of natural metabolites, biosynthesized in response to biotic and abiotic stress for surviving under extreme conditions, like excessive variations in temperature, desiccation, ultraviolet degradation, water stress and exposure to herbivores. Several of these species may be considered a potential source of phytochemicals such as antioxidants and antimicrobials produced in response to the stress they are exposed.

*Aphyllocladus spartioides* WEDD. (Asteraceae: Mutisieae) known as ‘pular’, ‘tola branca’, ‘tojra tola’ is a native herb distributed in the South of Bolivia, North of Chile and Northwest of Argentina. Their synonyms are *Hyalis spartioides* WEDD. and *Plazia spartioides* WEDD. In the prepuna of Salta and Jujuy, pular grows between 1500 and 3000 masl. All parts of this herb have been traditionally used for different purposes: infusion of stems and leaves as digestive stimulant, treatment of digestive infections and bone pain caused by cold or rheumatic diseases. Ethanolic extracts have been used topically to treat strains or sprains. Among these, stems and leaves of the plant are used in the artisanal production of regional foods with maize namely maiz puimado, tostado, and ulpada.

Despite these traditional uses, the existing information on chemical composition and biological potential of *A. spartioides* is scarce, being reported only few studies about the genus *Aphyllocladus*. Previous investigations on the chemical composition of *Aphyllocladus* revealed the presence of 5-methylcoumarin derivatives in *A. denticulatus*. In another study with *A. denticulatus*, aphyllocladine, lupeonone, lupeol, and apigenin were identified. Most recently, 80 volatile compounds were quantified in the essential oil (EO) of *A. spartioides*, α-pinene was reported as the main one.
Spathulenol, α-cadinol, γ- and δ-cadinene were found in important amounts. No significant chemical variability was found in the composition of volatile compounds within the studied populations of Maimara and Juella (Jujuy-Argentina). Regarding their biological potential, the scavenging effect of A. spartioides H2O extracts against DPPH proved to be promising. According with our knowledge, there are no reports about the phenolic composition of A. spartioides and few ones about its volatile compounds. The aims of this study were to investigate the volatile profile of the EO from A. spartioides aerial parts and their antibacterial potential. Additionally, the phenolic profile of hydroethanolic extract (HE) and decoction extract (DE) was evaluated, as well as their antioxidant, antibacterial, and α-glucosidase inhibition activities for further possible exploitation as food additive or food supplement associated to their health-promoting qualities.

Results and Discussion

Extraction Yield

The yields obtained with both extraction procedures were 25.9 ± 1.9% and 24.4 ± 3.5% (from starting dry material) for HE and DE, respectively.

Several factors can affect the recovery of metabolites from natural materials. Variables like temperature, time, solvent and solvent to plant ratio influenced the phytochemicals yields in the extractions from diverse plant materials. In this work, the yield obtained using EtOH/H2O with sonication was similar to the yield in the extraction with boiling water for a short period (P-value > 0.05). These results were mainly due to polarity of the compounds present in the plant material extracted with the polar solvents and to time/temperature conditions.

The yield of EO isolated by distillation from the aerial parts was 0.85 ± 0.01% (v/w), based on the dry weight of the sample. This result is in accordance with previously reported yield for EO extraction in the same species.

Chemical Profile of the EO

Sixty-eight compounds were identified and quantified in the EO of A. spartioides, representing 99.1% of the oil (Table 1). The EO compounds present in higher amount were α-pinene (37.8%), δ-cadinene (8.1%), γ-cadinene (4.1%), and (E)-caryophyllene (3.5%) (Table 1). These results are in accordance with those previously reported about the volatile composition of EO from A. spartioides, being α-pinene, γ- and δ-cadinene, spathulenol, and α-cadinol reported as the main compounds. In other study, α-thujone, β-thujone, sabine and 1.8-cineole were reported as the main components in A. decussatus EOs.

Phenolic Compounds. Phenolic profile of DE and HE is shown in Table 2. The HPLC-DAD analysis (Fig. 1) allowed the determination of 18 phenolic compounds, that comprised eleven free phenolic acids and derivatives (1 – 8, 12, 13, and 18) and seven flavonoids (9 – 11 and 14 – 17) (Table 2). Both extracts revealed similar profile, nevertheless they showed different contents (Table 2).

The phenolic contents ranged between 120143.2 and 15979.6 µg/g (of dried extract) for DE and HE, respectively (Table 2). With respect to flavonoids, isorhamnetin-3-O-glucoside (15) was the most abundant in HE and DE (ca. 27 and 32% of the phenolic determined, respectively), followed by isorhamnetin-3-O-rutinoside (16) (ca. 20 and 22% of the total phenolic contents, respectively). Other minor flavonoids in both extracts were quercetin and kaempferol derivatives (Table 2).

Isorhamnetin and derivatives are abundant flavonols in herbal medicinal plants, which are frequently used in the prevention and treatment of cardiovascular diseases; they can protect endothelial cells from injury caused by oxidized low-density lipoprotein, decrease blood pressure and alleviate the damage of ischemia reperfusion to ventricular myocytes. Regarding phenolic acids, they corresponded to ca. 41 and 34% of the total phenolic contents in HE and DE, respectively. Phenolic acid derivative 6 and 8 were the major ones in both extracts (Table 2); other phenolic acids and derivatives including caffeic acid, 3-O-cafeoylquinic acid, and 5-O-cafeoylquinic acid were quantified in minor proportions in both extracts.

The hydroxycinnamic acids normally present in fruits and vegetables are caffeic, chlorogenic, and caffeoylquinic acids. Other derivatives can be found in different amount in several plant samples. The antioxidant properties of these phytochemicals are well-known; in the human body, hydroxycinnamic acids can help to suppress the oxidative damage by acting directly on reactive species or by protecting the endogenous antioxidant system.

Antibacterial Activity

Aphyllocladus spartioides polar extracts and EO were tested against four Gram-positive and three Gram-
<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Rf</th>
<th>tR</th>
<th>[%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Thujene</td>
<td>932</td>
<td>5.2</td>
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<tr>
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<td>α-Pinenene</td>
<td>940</td>
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<td>Limonene</td>
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<td>1.92 ± 0.00</td>
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<tr>
<td>9</td>
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<td>1034</td>
<td>7.8</td>
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<td>4.11 ± 0.10</td>
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<td>8.06 ± 0.82</td>
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<tr>
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<td>trans-Cadina-1,4-diene</td>
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<td>23.6</td>
<td>0.51 ± 0.01</td>
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<tr>
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<td>α-Cadinene</td>
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<td>0.63 ± 0.02</td>
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<tr>
<td>50</td>
<td>α-Calacorene</td>
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<td>β-Atlantol</td>
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<td>0.46 ± 0.01</td>
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</table>
negative bacterial strains, MICs and MLCs were determined (Table 3); reference values for gentamicin (control) and α-pinene[18][19] were included for comparison purposes.

The EO was more active against Gram-positive than Gram-negative bacteria; the lowest MIC, 2.0 μl/ml was measured against B. cereus followed by S. aureus and M. luteus (both with MIC = 3.9 μl/ml) (Table 3). Additionally, EO revealed higher bactericidal activity against S. aureus (MLC = 31.1 μl/ml).

The biological activities of the EOs are often attributed to the major compounds present in the oils.[20] Regarding α-pinene, it was widely investigated as antibacterial agent (Table 3), being reported among the main responsible for the biological activities observed in EOs of Rosmarinus officinalis.[18][19]

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Table 1. (cont.)

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Rf</th>
<th>tR</th>
<th>[%]c</th>
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<td>0.41 ± 0.00</td>
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<td>Muurola-4,10(14)-dien-1β-ol</td>
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<td>Caryophylla-4(12),8(13)-dien-5β-ol</td>
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<tr>
<td>Σ</td>
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</table>

a All components were identified by comparison of their RI (retention indices) and mass spectra with literature data, the MS library (NBS 75K, NIST98), and a spectra library built up from pure substances and components of known oils; experimental RI on HP5-MS capillary column in reference to C7-C24 n-alkanes; the compounds are listed in order of elution. b tR, Retention time. c Relative percentages of peak area as means of two determinations ± standard deviation. d Oil component present in higher amount.

Table 2. Phenolic compounds in Aphyllocladus spartioides dried extracts

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Concentration [μg/g]a and percentage [%]</th>
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</thead>
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<td>HE</td>
</tr>
<tr>
<td>1</td>
<td>3-O-Caffeoylquinic acid</td>
<td>1629.4 ± 10.1 (1.0%)</td>
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<tr>
<td>2</td>
<td>5-O-Caffeoylquinic acid</td>
<td>6430.5 ± 34.8 (4.0%)</td>
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<tr>
<td>3</td>
<td>Caffeic acid</td>
<td>4565.2 ± 13.9 (2.9%)</td>
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<tr>
<td>4</td>
<td>Phenolic acid derivative 1</td>
<td>390.5 ± 3.7 (0.2%)</td>
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<tr>
<td>5</td>
<td>Phenolic acid derivative 2</td>
<td>4210.9 ± 4.0 (2.6%)</td>
</tr>
<tr>
<td>6</td>
<td>Phenolic acid derivative 3</td>
<td>16667.6 ± 58.6 (10.4%)c</td>
</tr>
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<td>7</td>
<td>Phenolic acid derivative 4</td>
<td>2032.6 ± 5.3 (1.3%)</td>
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<td>Phenolic acid derivative 5</td>
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<td>Quercetin-3-O-galactoside</td>
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<td>Quercetin-3-O-gluconoside</td>
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</tr>
<tr>
<td>11</td>
<td>Quercetin-3-O-rutinoside</td>
<td>3014.4 ± 1.1 (1.9%)</td>
</tr>
<tr>
<td>12</td>
<td>Phenolic acid derivative 6</td>
<td>5556.6 ± 49.1 (3.5%)</td>
</tr>
<tr>
<td>13</td>
<td>Phenolic acid derivative 7</td>
<td>5571.5 ± 16.3 (3.5%)</td>
</tr>
<tr>
<td>14</td>
<td>Kaempferol-3-O-rutinoside</td>
<td>878.9 ± 1.4 (0.6%)</td>
</tr>
<tr>
<td>15</td>
<td>Isorhamninetin-3-O-glucoside</td>
<td>43167.3 ± 97.6 (27.0%)</td>
</tr>
<tr>
<td>16</td>
<td>Isorhamninetin-3-O-rutinoside</td>
<td>32203.4 ± 8.4 (20.2%)</td>
</tr>
<tr>
<td>17</td>
<td>Quercetin derivative</td>
<td>1002.5 ± 1.5 (0.6%)</td>
</tr>
<tr>
<td>18</td>
<td>Phenolic acid derivative 8</td>
<td>705.4 ± 0.3 (0.4%)</td>
</tr>
<tr>
<td>Σb</td>
<td></td>
<td>159799.6</td>
</tr>
</tbody>
</table>

a Mean concentration in dry extract ± standard deviation of three assays. b Sum of the determined phenolic compounds. c Phenolic compounds present in higher amounts.
By the analysis of the obtained results, the HE and DE revealed bacteriostatic and bactericidal effect against all Gram-positive bacteria and none effect was observed for the tested concentrations against Gram-negative bacteria. HE was more active, revealing the lowest MIC and MLC values (Table 3). *Staphylococcus aureus* and *M. luteus* were the most sensitive strains (with MIC/MLC = 0.3 – 0.6/5.0 for HE and MIC/MLC = 2.5 – 5.0/10.0 for DE, respectively).

In the references, MICs were reported in % (v/v), μl/ml or mg/ml; for comparison it can be assumed that essential oils have the same density as H2O.\(^{[20]}\)

Comparing EO and polar extracts, HE revealed high bacteriostatic effect against the tested strains; however, slight differences were found in the lethal activity (Table 3). With DE, the bacteriostatic effect was only observed against *S. aureus* and *M. luteus*. In agreement with previous studies with plant extracts, Gram-positive bacteria were more sensitive, mainly with HE.\(^{[1][21]}\)

Considering that for plant extracts, strong activity was reported for MICs = 0.05 – 0.5 mg/ml, moderate activity for MICs = 0.6 – 1.5 mg/ml and weak activity above 1.5 mg/ml,\(^{[22]}\) we can conclude that HE possess strong antibacterial activity against Gram-positive strains.

*Staphylococcus aureus*, a recognized pathogen in humans, can cause a range of diseases in man, ranging from sub-clinical inflammation to severe infections;\(^{[23]}\) the bacterium readily acquires resistance against all classes of antibiotics. Additionally, *M. luteus* has been implicated in a variety of infections, including meningitis, endocarditis, septic arthritis and central nervous system infections in immunocompromised hosts.\(^{[24]}\)

Many plant extracts and EOs possess in vitro antimicrobial properties linked to their content of secondary metabolites, including terpenoids, alkaloids or flavonoids.\(^{[25 – 27]}\) However, if plant extracts and oils

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**Table 3. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of Aphyllocladus spartioides extracts (mg/ml) and essential oil (μl/ml)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample extract</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HE</td>
<td>MIC/MLC</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>0.6/5.0</td>
<td>5.0/10.0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.3 – 0.6/5.0</td>
<td>5.0/10.0</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>0.6/&gt;&gt; 5.0</td>
<td>10.0/&gt;&gt; 10.0</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td>0.3 – 0.6/5.0</td>
<td>2.5 – 5.0/10.0</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>&gt;&gt; 5.0/nq</td>
<td>&gt;&gt; 10.0/nq</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>&gt;&gt; 5.0/nq</td>
<td>&gt;&gt; 10.0/nq</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>&gt;&gt; 5.0/nq</td>
<td>&gt;&gt; 10.0/nq</td>
</tr>
</tbody>
</table>

HE: Hydroethanolic extract; DE: decoction extract. EO: Essential oil. \(^{a}\) Results are from three independent experiments performed in duplicate; when different MIC values were obtained a range of values is presented. \(^{b}\) *S. aureus* ATCC 25923 and *E. coli* ATCC 35218.\(^{[18]}\) \(^{c}\) *S. aureus*, *B. cereus* and *P. aeruginosa* were strains from Type Culture Collection;\(^{[19]}\) nq, not quantified.

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**Figure 1.** HPLC/DAD Phenolic profile of HE (detection at 320 nm). The identity of the numbered compounds is presented in Table 2.
are to be used for medicinal purposes or food preservation, issues of safety and toxicity will need to be addressed.\textsuperscript{[27]}

**Antioxidant Activity.** The HE and DE were tested for scavenging activity against DPPH\textsuperscript{2}, 'NO, and O\textsuperscript{2-} radicals (Fig. 2 and Table 4). Both extracts exhibited a dose-dependent activity against DPPH\textsuperscript{2} (Fig. 2a), although HE (IC\textsubscript{50} = 78.7 \mu g/ml) displayed slightly better activity than DE (IC\textsubscript{50} = 81.0 \mu g/ml) (Table 4). Furthermore, both extracts proved to be less active then the control Trolox (IC\textsubscript{50} = 34.3 \mu g/ml).

Subsequently, the extracts were also tested against 'NO and O\textsuperscript{2-}. HE and DE showed notable concentration-dependent effects. DE proved to be more active (IC\textsubscript{50} = 20.3 \mu g/ml) against O\textsuperscript{2-} than HE (IC\textsubscript{50} = 28.1 \mu g/ml) (Fig. 2b and Table 4). In respect to 'NO, the results revealed that the HE (IC\textsubscript{50} = 206.0 \mu g/ml) was more active than DE (IC\textsubscript{50} = 229.7 \mu g/ml) (Fig. 2c, Table 4).

The results obtained showed that *A. spartioides* extracts possess a promising antioxidant potential as free radical scavengers. This activity may be associated with their high level of flavonoids and their glycosides (like isorhamnetin derivatives among others) and phenolic acids, especially hydroxycinnamic acids derivatives that have attracted considerable attention due to its biological and pharmaceutical activities.\textsuperscript{[17][28]} Apart from the determined compounds, the contribution to the observed activity of other metabolites such as organic acid cannot be ignored.\textsuperscript{[29]}

**Figure 2.** Scavenging activity of polar extracts against a) DPPH\textsuperscript{2}, b) superoxide (O\textsuperscript{2-}) and c) nitric oxide ('NO) radicals. d) \alpha-glucosidase inhibition of *Aphyllocladus spartioides* polar extracts. Values show mean ± standard deviation of three experiments performed in triplicate.

**Table 4.** Antiradical activity and \alpha-glucosidase inhibition of hydroethanolic extract, decoction extract and controls

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC\textsubscript{50} [\mu g/ml]</th>
<th>DPPH\textsuperscript{2}</th>
<th>'NO</th>
<th>O\textsuperscript{2-}</th>
<th>\alpha-glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE</td>
<td>78.7 ± 0.4\textsuperscript{b}</td>
<td>206.0 ± 9.5\textsuperscript{a}</td>
<td>28.1 ± 1.1\textsuperscript{a}</td>
<td>181.4 ± 2.1\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>DE</td>
<td>81.0 ± 0.4\textsuperscript{a}</td>
<td>229.7 ± 13.8\textsuperscript{a}</td>
<td>20.3 ± 0.2\textsuperscript{b}</td>
<td>1356.4 ± 117.9\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>Trolox</td>
<td>34.3 ± 0.2\textsuperscript{c}</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>284.0 ± 35.5\textsuperscript{b}</td>
</tr>
</tbody>
</table>

IC\textsubscript{50}: Minimum concentration of sample which reduces the absorbance 50%; Trolox, antioxidant standard; acarbose, reference compound for the \alpha-glucosidase inhibition; \textsuperscript{a–c} means with different superscripts are significantly different (P value < 0.05).
Conclusions

The chemical and biological potential of \textit{A. spartioides} collected in Hornillos, Jujuy (Argentina) is dependant on the use of different techniques of extraction. \textit{Aphyllolcadius spartioides} studied here is a rich source of natural phytochemicals, such as isorhamnetin derivatives, different phenolic acids, \(\alpha\)-pinene and cadinene among others. HE revealed the higher contents in phenolic compounds and, in a general way, proved to be the extract with better biological potential. Also the essential oil of \textit{A. spartioides} showed in vitro antibacterial activity and these results contribute to support the large diffusion of antimicrobial activity of essential oils. However, more studies of non-volatiles compounds in other places of growth of the species are necessary to establish the composition and homogeneity of plant populations.

Also more comprehensive studies of the chemical composition and selected toxicological assays of these extracts should be carried out in the future to ensure their safety for pharmaceutical incorporations or food supplementation.

Experimental Section

Abbreviations

EO: essential oil; DE: decoction extract; HE: hydroethanolic extract; \textit{Trolox}: (±)-6-hydroxy-2,5,7,8-tetramethylchomane-2-carboxylic acid; DPPH\(^{+}\): 1, 1-diphenyl-2-picrylhydrazyl; NADH: \(\beta\)-nicotinamide adenine dinucleotide reduced form; PMS: phenazine-metho sulfate; NBT: nitrotetrazolium blue chloride; DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); AChE: acetylcholinesterase; ATCl: acetlythiocholine iodide; BuChE: butrylcholinesterase; PNP-G: 4-nitrophenyl-\(\alpha\)-o-glucopyranoside; SNP: sodium nitroprusside dehydrate; MHB: Mueller Hinton Broth; MHA: Mueller Hinton Agar; MIC: minimum inhibitory concentration; MLC: minimum lethal concentration; \(IC_{50}\): minimum concentration of sample which reduces the absorbance about 50%.

Standards and Reagents

The standard compounds were purchased from various suppliers: caffeeic acid and \textit{Trolox} were from \textit{Sigma–Aldrich} (St. Louis, MO, USA); 5-O-cafeoylquinic acid, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, isorhamnetin-3-O-glucoside, isorhamnetin-3-O-rutinoside, were from \textit{Extra Synthèse} (Genay, France). DPPH\(^{+}\), sodium pyruvate, NADH, PMS, NBT, DTNB, sulphamidine, AChE from electric eel (type VI-s, lyophilized powder), ATCl, BuChE from equine serum (lyophilized powder), 5-butyrylthiocholine chloride, \(\alpha\)-glucosidase (type I from baker’s yeast), PNP-G, SNP, MeOH, gentamicin and \(n\)-alkanes standard mixture (\(C_7 - C_{24}\)) were purchased from \textit{Sigma–Aldrich} (St. Louis, MO, USA). Acarbose was from \textit{Blueharma Genéricos} (Coimbra, Portugal). \(N\)-(1-Naphthyl)ethylenediamine dihydrochloride, EtOH and potassium dihydrogen phosphate were obtained from \textit{Merck} (Darmstadt, Germany). Hydrochloric and ortho-phosphoric acid were purchased from \textit{Panreac} (Barcelona, Spain). MHB and MHA media were purchased from \textit{Liofilchem} (Teramo, Italy). Water was deionized using a \textit{Millipore} water purification system (\textit{Millipore}, Bedford, MA, USA).

Plant Samples

Aerial parts of eight specimens of \textit{A. spartioides} (400 – 600 g each one) were collected in April of 2013, from a homogenous tojra-tola population\(^{[6]}\) at Hornillos (23°39′20.2″ S 65°25′59.4″ O, 2400 m), Province of Jujuy (Argentina) with 0 – 10% open flowers.

The plant material was identified by Prof. Osvaldo Ahumada (National University of Jujuy, Argentina) and Prof. Gustavo Giberti (National University of Buenos Aires, Argentina). Voucher specimen (HN1310) was deposited with the Herbarium of PRONOÁ-UNJu (Faculty of Engineering from National University of Jujuy, Argentina) and Herbarium BAF (Buenos Aires Farmacobotánica). The plant material was dried at r.t. during
1 week, ground to powder in a blender (mean particle size < 2 mm) and stored at −20 °C until required.

Extracts Preparation

EO was extracted from two samples of approximately 500 g of dried plant material by steam distillation for 2.5 h using a Clevenger-type apparatus. The collected oil samples were dried (Na$_2$SO$_4$) and stored at 4 °C until analysis.[32] The EO content was determined volumetrically on a dried weight basis, obtaining approximately the same yield.

*Aphyllocladus spartioides* aerial parts were powdered and sieved in order to obtain a mean particle size lower than 910 μm and used for the preparation of two different extracts. HE was obtained according to Celaya, Vituro, Silva, and Moreno[11] with minor modifications, 1 g of dried leaves were extracted by sonication at 40 °C with 20 ml of EtOH/H$_2$O 70:30 during 20 min. The extract obtained was evaporated under reduced pressure and kept at −20 °C until further analysis. For DE, 5 g of dried leaves were boiled with 100 ml of H$_2$O for 10 min.[1] The resulting extract was filtered through a Büchner funnel, frozen and lyophilized; these extracts were kept in a desiccator, in the dark, until analysis. Extractions were carried out in triplicate.

Volatile Compounds

EO composition was analyzed by GC-IT-MS and GC-FID using a previously described procedure.[32] GC-IT-MS analysis was carried out on a Hewlett Packard GC 6890/MDS 5972 apparatus equipped with a HP-5 MS Agilent Technologies column (30 m × 0.25 mm; 0.25 μm) and a Saturn GC/MS workstation software version 6.8. The injector port was heated to 300 °C and the samples were injected using a split ratio of 1:40, with helium C-60 as the carrier gas at constant flow rate of 1.5 ml/min. The oven temp. program was set at 60 °C for 5 min, increasing by 6 °C/min to 250 °C and then 5 °C/min to 300 °C. All mass spectra were acquired in electron impact (EI) mode. Ionization was turned off for the first 4 min to avoid solvent overloading. The settings on the ion trap detector were programmed as follows: transfer line, manifold and trap temp. at 300, 50, and 180 °C, resp. The mass ranged from 50 to 600 m/z with a scan rate of six scans per second. The emission current was 50 μA, and the electron multiplier was set in relative mode to the auto tune procedure. The maximum ionization time was 25 000 Is, with an ionization storage level of 35 m/z. The injection volume was 1 μl, and the analysis was performed in the full-scan mode.

The components of the EO were identified by comparison of their retention indices (RI) and mass spectra with those from the literature data,[33 – 35] those recorded in the MS library (NBS 75K, NIST98), and those of a spectra library built up from pure substances and components of known oils. The RI was determined relative to a series of n-alkanes (C$_7$ – C$_{26}$).

Quantitative analysis of the EO was carried out using a GC-FID Konik 3000G equipped with a flame ionization detector (FID) and a HP-5 fused silica capillary column (30 m × 0.25 mm with 0.25 μm). The oven temp. was set at 60 °C for 5 min, increasing by 6 °C/min to 250 °C and then 5 °C/min to 300 °C. The injector and detector temp. were 300 and 310 °C, resp. Helium C-60 (Gasin, Portugal) was used as a carrier gas at a constant flow rate of 1.5 ml/min, and 1 μl samples were injected using a split ratio of 1:20. The percentage composition of the products was calculated by normalization of the GC peak areas without response factors.

Phenolic Compounds

Polar extracts were dissolved in MeOH (30 mg/ml) and filtered through a PTFE membrane (0.45 μm). Resulting dissolutions were analyzed in a HPLC/DAD (Gilson) following a previous described methodology.[31] Twenty μl of each polar extract were analyzed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size; Waters, Milford, MA) column. The solvent system used was a gradient of H$_2$O/HCOOH 19:1 (A) and MeOH (B), starting with 5% MeOH and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson diode array detector (DAD). Spectral data from peaks were accumulated in the range 200 – 400 nm, and chromatograms were recorded at 280, 320, and 350 nm. The data were processed on Unipoint System software (Gilson Medical Electronics, Villiers-le-Bel, France). Compounds were identified by comparing the retention times and UV/VIS spectra with those of authentic standards and with the characteristic absorption spectra in our database; quantification was achieved by measuring the absorbance recorded in the chromatograms relative to the external standards. This procedure was performed in triplicate. 3-O-Caffeoylquinic acid and phenolic acid derivatives were quantified as 5-O-
caffeoylquinic acid; quercetin derivative 1 was quantified as quercetin-3-O-glucoside.

In Vitro Antibacterial Activity

The study included seven bacteria strains: *Staphylococcus aureus* ATCC 25923 (control strain), *S. aureus* (ATCC 20231), *Bacillus cereus* (ATCC 31), *Micrococcus luteus* (ATCC 20030), *Salmonella typhimurium* (ATCC 43971), *Escherichia coli* (ATCC 30083) and *Pseudomonas aeruginosa* (ATCC 50071), these species were selected due to their great importance in foods and medicine. Gentamicin MIC and MLC for *S. aureus* (ATCC 25923) were determined as quality control. Cultures were obtained from the Department of Microbiology, Faculty of Pharmacy, Porto University (Portugal).

MLCs were determined by employing broth microdilution methods following a described procedure, based on the CLSI guidelines, reference documents M07-A8 and M100-S19, with minor modifications. The MIC of extracts and EOs were determined by two-fold serial dilution method, in 96-well plates. The initial concentration was 5.0 mg/ml of dry matter for HE, 10.0 mg/ml of dry matter for DE and 62.5 μl/ml for EO. The maximum DMSO concentration did not exceed 2.5% (v/v). Positive control wells contained microorganisms without antibiotics. Sterility and positive controls in MHB medium alone and with 2.5% of DMSO (v/v) were included.

The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 18 – 24 h and MIC was determined as the lowest concentration of extracts inhibiting the visual growth of the test culture on the microplate. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results.

MLCs for EO and extracts were determined by removing 20 μl of all wells showing no visible growth on MHA plates. The plates were incubated at 37 °C. The MLC was defined as the lowest concentration showing 100% growth inhibition after 18 – 24 h of incubation.

Antioxidant Activity

The HE and DE were used for the screening of the antioxidant activity. Spectrophotometric microassays were performed in a Multiskan Ascent plate reader (Thermo, Electron Corporation). For each extract, a dilution series (seven different concentrations) was prepared in a 96-well plate. For each radical, three independent assays were performed in triplicate.

DPPH’ Assay. The disappearance of DPPH’ was monitored spectrophotometrically at 515 nm, following a described procedure. The antioxidant Trolox was used as reference standard.

Nitric Oxide Assay. The scavenging activity was determined following a described procedure. The chromophore formed with Griess reagent was read at 562 nm.

Superoxide Radical Assay. All components were dissolved in phosphate buffer (19 mM, pH 7.4). Superoxide radicals were generated by the NADH/PMS system, as previously reported. The effect of DE and HE on superoxide radical-induced reduction of NBT was monitored at 562 nm.

The concentration of extract that reduces 50% of the free-radical concentration (IC₅₀ value, μg/ml) was calculated through regression from the percentages of inhibition. All data were recorded as mean ± standard deviation of triplicate determinations. Mean values were compared using two way ANOVA and post-hoc Tukey test to determine differences with statistical significance. Differences were considered significant for p-value < 0.05. Statistical analysis was carried out using Graphpad Prism 5 Software (San Diego, CA, USA).

α-Glucosidase Inhibitory Activity. α-Glucosidase inhibitory activity was assessed according to a method previously described. Briefly, each well contained 100 μl of PNP-G (2 mM) in potassium phosphate buffer (100 mM, pH 7.0) and 20 μl of the extract in buffer. The reaction was initiated by the addition of 100 μl of the enzyme solution (56.6 mU/ml). The plates were incubated at 37 °C for 10 min. The absorbance of 4-nitrophenol released from PNP-G at 400 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity. For each extract (HE and DE), three independent assays were performed in triplicate. Acarbose was used as positive control. Seven different concentrations were tested and IC₅₀ value (μg/ml) was calculated through regression from the percentages of inhibition. Mean values were compared using two way ANOVA and post-hoc Tukey test to determine differences with statistical significance.

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