

## Antioxidant phenylacetic acid derivatives from the seeds of *Ilex aquifolium*

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Reversed-phase preparative HPLC analysis of the methanol extract of the seeds of *Ilex aquifolium* afforded two antioxidant phenylacetic acid derivatives, 2,4-dihydroxyphenylacetic acid (**1**) and 2,4-dihydroxyphenylacetic acid methyl ester (**2**). The structures were determined by spectroscopic methods. In the DPPH assay for antioxidant activity, the  $IC_{50}$  values of **1** and **2** were  $1.50 \times 10^{-3}$  and  $2.55 \times 10^{-3}$  mg mL<sup>-1</sup>, respectively, compared to  $2.88 \times 10^{-5}$  mg mL<sup>-1</sup> of quercetin, a natural antioxidant.

**Keywords:** *Ilex aquifolium* (*Aquifoliaceae*), phenylacetic acid derivatives, antioxidant, DPPH

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*Ilex aquifolium* L. (*Aquifoliaceae*), commonly known as 'English holly' or 'holly', a decorative tree for Christmas festivities, is native to the United Kingdom and other countries in Europe (e.g., Mediterranean countries), temperate Asia, and Africa (1, 2). Various parts of 'holly' are still today included in traditional medicinal preparations to treat liver, stomach and intestinal cancers, dropsy, fever, gout, jaundice, malaria, warts, swelling and tumours (3). It is also known to have diuretic, emetic, emollient, purgative and tonic properties. Previous phytochemical investigations on *I. aquifolium* revealed the presence of various secondary metabolites, including sterols and terpenoids (4–7), cyanogenic glucosides (9, 10), anthocyanins (11, 12) and flavonoids (5). However, most of these investigations were primarily concerned with leaves, stems, flowers and fruits, but not

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seeds. As part of our on-going search for natural antioxidants from higher plants (13–21), we now report on the isolation, structure determination and antioxidant properties of two phenylacetic acid derivatives from the seeds of *I. aquifolium*.

## EXPERIMENTAL

### *General procedures*

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV-Vis spectrometer (Agilent, Germany). CIMS (Chemical Ionisation Mass Spectrometry) analyses were performed at the EPSRC Central Mass Spectroscopy Facility in Swansea, UK, on a Micromass Quattro II triple quadrupole instrument (Waters, UK) in chemical desorption mode using ammonia as CI gas. Mass accuracy was within 0.4 Da. CI source temperature was 170 °C and electron energy was 59 eV. NMR spectra were obtained in CD<sub>3</sub>OD using Bruker AC250 (250 MHz for <sup>1</sup>H and 62.5 MHz for <sup>13</sup>C) (Bruker, UK) and/or Jeol LA-300 NMR (300 MHz for <sup>1</sup>H and 75.0 MHz for <sup>13</sup>C) (Jeol, Japan) spectrometers. Chemical shifts  $\delta$  were in ppm. The HMQC (Heteronuclear Multi Quantum Coherence) method used the BIRD (Bilinear Rotation Decoupling) pulse sequence and the HMBC (Heteronuclear Multiple Bond Coherence) experiment had a 70 ms long-range coupling delay. Spectra were recorded with a probe temperature of 25 °C. Preparative reversed-phase HPLC was carried out in a Dionex 580 HPLC system Dionex Corporation, USA coupled with a UVD340S photo-diode-array detector and Gina50 autosampler (GynkoteK, USA). A Luna C<sub>18</sub> preparative column (21.2 x 250 mm, 10  $\mu$ m) from Phenomenex (UK) was used. A Sep-Pak Vac (Waters, USA) 10 g cartridge was used for pre-HPLC fractionation of the MeOH extract. 2,2-Diphenyl-1-picrylhydrazyl (molecular formula C<sub>18</sub>H<sub>12</sub>N<sub>5</sub>O<sub>6</sub>, DPPH) and quercetin (quercetin dihydrate, > 99%) were purchased from Fluka (UK) and were used without further purification. Precoated aluminium sheets silica gel 60 F<sub>254</sub> (0.25 mm thickness) pre-coated TLC plates (Merck, Germany) were used. TLC mobile phases were *n*-hexane-ethylacetate (EtOAc) mixtures of various proportions, *e.g.*, 5% EtOAc in *n*-hexane, 10% EtOAc in *n*-hexane, *etc.*

### *Plant material*

The seeds of *Ilex aquifolium* (*Aquifoliaceae*) were purchased from B & T World Seeds Sarl, France. A voucher specimen (PH004006 SDS) has been deposited in the herbarium of Plant and Soil Science Department, University of Aberdeen, Scotland, UK.

### *Extraction and isolation*

Dried ground seeds of *I. aquifolium* (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane (DCM) and methanol (1.1 L each). All three extracts were concentrated using a rotary evaporator at a temperature not exceeding 50 °C. From the preliminary thin layer chromatographic analysis, it was obvious that the *n*-hexane and DCM extracts contained predominantly long-chain alkanes, and fatty alcohols, acids and esters, and therefore were not subjected to further phytochemical purification. The me-

thanol extract was subjected to solid-phase extraction on a Sep-Pak C<sub>18</sub> cartridge (10 g), Waters, USA eluted with a step gradient using 30, 60, 80 and 100% MeOH in water (200 mL each). The Sep-Pak fraction eluted with 30% MeOH in water was analyzed by preparative reversed-phase HPLC (mobile phase: 0–25 min 30% MeOH in water, isocratic; 25–50 min linear gradient from 30 to 100% MeOH in water; 20 mL min<sup>-1</sup>, detection at 220 and 254 nm) to yield two phenylacetic acid derivatives: 2,4-dihydroxyphenylacetic acid [**1**, yield 0.02%; *t*<sub>R</sub> = 7.64 min; UV  $\lambda_{\text{max}}$  in MeOH: 278 nm; CIMS *m/z*: 186 [M+NH<sub>4</sub>]<sup>+</sup>; <sup>1</sup>H and <sup>13</sup>C NMR in Table I] and 2,4-dihydroxyphenylacetic acid methyl ester [**2**, yield 0.08%; *t*<sub>R</sub> = 11.71 min; UV  $\lambda_{\text{max}}$  in MeOH: 278 nm, CIMS *m/z*: 200 [M+ NH<sub>4</sub>]<sup>+</sup>; <sup>1</sup>H and <sup>13</sup>C NMR in Table I]. Both **1** and **2** were obtained as amorphous solids.

Table I. <sup>1</sup>H and <sup>13</sup>C NMR data of compounds **1** and **2**<sup>a</sup>

Position	<sup>1</sup> H NMR Chemical shift ( $\delta$ ppm)		<sup>13</sup> C NMR Chemical shift ( $\delta_{\text{C}}$ , ppm)	
	<b>1</b> <sup>b</sup>	<b>2</b> <sup>c</sup>	<b>1</b> <sup>d</sup>	<b>2</b> <sup>e</sup>
1	–	–	113.7	113.7
2	–	–	158.7	158.7
3	6.29 d (2.4)	6.27 d (2.4)	103.3	103.3
4	–	–	157.5	157.5
5	6.23 dd (2.4, 8.3)	6.22 dd (2.4, 8.3)	107.4	107.4
6	6.89 d (8.3)	6.87 d (8.3)	132.4	132.4
7	3.50 s	3.49	35.9	35.8
8	–	–	175.5	175.2
OMe	–	3.65 s	–	52.3

<sup>a</sup> Coupling constant *J* in Hz in parentheses.

<sup>b</sup> Spectrum obtained in CD<sub>3</sub>OD (250 MHz).

<sup>c</sup> Spectrum obtained in CD<sub>3</sub>OD (300 MHz).

<sup>d</sup> Spectrum obtained in CD<sub>3</sub>OD (62.5 MHz).

<sup>e</sup> Spectrum obtained in CD<sub>3</sub>OD (75 MHz).

#### Antioxidant assay (DPPH assay)

DPPH was used in this assay to assess the free radical scavenging (antioxidant) properties of **1** and **2** (21, 22). Quercetin, a well known natural antioxidant, was used as a positive control. DPPH solution in MeOH (80  $\mu\text{g mL}^{-1}$ ) was used. IC<sub>50</sub> (50% inhibitory concentrations) of **1**, **2** and quercetin was evaluated with respect to MeOH as a negative control.

*Qualitative assay.* – Test compounds (**1** and **2**, 1 mg mL<sup>-1</sup> in MeOH) were applied on a TLC plate and sprayed with DPPH solution using an atomizer. It was allowed to develop for 30 min. White spots against a pink background indicated the antioxidant activity. The same procedure was followed with quercetin.

**Quantitative assay.** – For the quantitative assay, stock solutions of compounds **1** and **2** were prepared in MeOH to achieve a concentration of 1 mg mL<sup>-1</sup>. Dilutions were made to obtain concentrations of 5 × 10<sup>-2</sup>, 5 × 10<sup>-3</sup>, 5 × 10<sup>-4</sup>, 5 × 10<sup>-5</sup>, 5 × 10<sup>-6</sup>, 5 × 10<sup>-7</sup>, 5 × 10<sup>-8</sup>, 5 × 10<sup>-9</sup>, 5 × 10<sup>-10</sup> mg mL<sup>-1</sup>. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance of these solutions was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control (quercetin).

## RESULTS AND DISCUSSION

Reversed-phase preparative HPLC analysis of the methanol extract of the seeds of *Ilex aquifolium* resulted in the isolation of two antioxidant phenylacetic acid derivatives, 2,4-dihydroxyphenylacetic acid (**1**) and 2,4-dihydroxyphenylacetic acid methyl ester (**2**) (Fig. 1). The structures of these compounds were elucidated by UV, MS, and 1D and 2D NMR spectroscopic analyses.

The UV absorption maxima of **1** and **2** at 278 nm indicated the presence of aromatic nucleus in these molecules. The common features of the <sup>1</sup>H NMR spectra of **1** and **2** (Table I) were the signals at δ (ppm) of 6.23/6.22, 6.29/6.27, 6.89/6.87 and 3.50/3.49, corresponding to a trisubstituted benzyl system present in these compounds. In addition to these signals, the <sup>1</sup>H NMR of **2** showed the signal for a methyl ester moiety (δ 3.65). The <sup>13</sup>C NMR spectra (Table I), with the exception of the signal (δ 52.3) attributable to a methyl ester group in **2**, displayed signals for six aromatic carbons including two oxygenated quaternary carbons (δ 158.7 and 157.5), a quaternary carbon (δ 113.7) and three methine carbons (δ 103.3, 107.4 and 132.4), and a methylene (δ 35.9/35.8) and acid/ester carbonyl (δ 175.5/175.2). From the <sup>1</sup>H and <sup>13</sup>C NMR data analysis, it was obvious that the only difference between **1** and **2** was the presence of a methyl ester group in **2**. The CIMS experiments displayed the pseudomolecular ions [M+NH<sub>4</sub>]<sup>+</sup> at *m/z* 186 and 200, respectively, for **1** and **2**, and confirmed that compound **2** had 14 mass units more than **1**, which

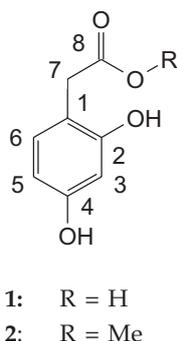


Fig. 1. Structures of phenylacetic acid derivatives (**1** and **2**) isolated from *I. aquifolium*.

was owing to the presence of the methyl ester group in **2**. For the unambiguous assignment of all  $^1\text{H}$  and  $^{13}\text{C}$  signals,  $^1\text{H}$ - $^{13}\text{C}$  HMQC and  $^1\text{H}$ - $^{13}\text{C}$  HMBC experiments were carried out on **2** (Fig. 2). The HMQC experiment confirmed the assignment of all methine, methylene and methyl groups. In the HMBC spectrum, the key  $^1\text{H}$ - $^{13}\text{C}$  long-range correlations were: from H-3 ( $\delta$  6.27),  $^3J$  to C-1 ( $\delta$  113.7) and C-5 ( $\delta$  107.4); from H-5 ( $\delta$  6.22),  $^3J$  to C-1 and C-3 ( $\delta$  103.3); from H-6 ( $\delta$  6.87),  $^3J$  to C-2 ( $\delta$  158.7), C-4 ( $\delta$  157.5) and C-7 ( $\delta$  35.8); from H<sub>2</sub>-7 ( $\delta$  3.49),  $^3J$  to C-2 and C-6 ( $\delta$  132.4),  $^2J$  to C-1 and C-8 ( $\delta$  175.2); from the methyl ester group ( $\delta$  3.65),  $^3J$  to C-8. Thus, the structures of these compounds were determined unequivocally as 2,4-dihydroxyphenylacetic acid (**1**) and 2,4-dihydroxyphenylacetic acid methyl ester (**2**). To our knowledge, this is the first report on the occurrence of phenylacetic acid derivatives in *I. aquifolium*, and also in the genus *Ilex*. While compound **1** was first reported as a constituent of the venom of the spider *Nephila maculata*, compound **2** was previously isolated from *Madhuca pasquieri* (23).

In the DPPH assay, compounds **1** and **2** showed moderate levels of free radical scavenging (antioxidant) activity compared to that of the positive control, quercetin. The  $IC_{50}$  values for **1**, **2** and quercetin were found to be  $1.5 \times 10^{-3}$ ,  $2.55 \times 10^{-3}$  and  $2.88 \times 10^{-5}$  mg mL $^{-1}$ , respectively. The antioxidant activity of **1** and **2**, like other natural phenolic antioxidants, *e.g.*, flavonoids (15), is a consequence of the presence of the phenolic moieties in the structures. The antioxidant activity of phenolic natural products is predominantly due to their redox properties, *i.e.*, the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, it could also be due to their metal chelation potential (15).

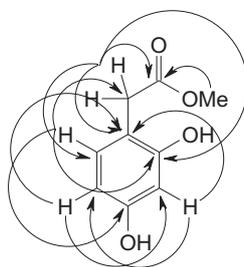


Fig. 2.  $^1\text{H}$ - $^{13}\text{C}$  long-range ( $^2J$  and  $^3J$ ) correlation observed in the HMBC spectrum of compound **2**.

## CONCLUSIONS

The free radical scavenging property of compounds **1** and **2**, which are present in good amounts in the seeds of *I. aquifolium*, may explain, at least to some extent, some of the traditional medicinal uses of this plant.

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## S A Ž E T A K

### Derivati fenilactene kiseline s antioksidativnim učinkom iz sjemenki biljke *Ilex aquifolium*

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Reverzno-faznom preparativnom HPLC analizom metanolnog ekstrakta sjemenki biljke *Ilex aquifolium* izolirana su dva derivata fenilactene kiseline s antioksidativnim učinkom, 2,4-dihidroksifenilactena kiselina (1) i metilni ester 2,4-dihidroksifenilactene kiseline (2). Njihove strukture određene su spektroskopskim metodama. U DPPH testu na antioksidativno djelovanje,  $IC_{50}$  vrijednosti spojeva 1 i 2 bile su  $1,50 \times 10^{-3}$  i  $2,55 \times 10^{-3}$  mg mL<sup>-1</sup>, dok je  $IC_{50}$  prirodnog antioksidansa kvercitina bila  $2,88 \times 10^{-5}$  mg mL<sup>-1</sup>.

*Ključne riječi:* *Ilex aquifolium* (*Aquifoliaceae*), derivati fenilactene kiseline, antioksidans, DPPH

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