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Irbic acid, a dicaffeoylquinic acid derivative from *Centella asiatica* cell cultures

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ABSTRACT

3,5-*O*-dicaffeoyl-4-*O*-malonilquinic acid (**1**) (irbic acid) has been isolated for the first time from cell cultures of *Centella asiatica* and till now it has never been reported to be present in the intact plant. Evidence of its structure was obtained by spectroscopic analyses (MS/NMR). Besides **1**, cell cultures produce also the known 3,5-*O*-dicaffeoylquinic acid, chlorogenic acid, and the triferulic acid **2** (4-*O*-8′/4′-*O*-8″-didehydrotriferulic acid). Biological activities were evaluated for compound **1**, which showed to have a strong radical scavenging capacity, together with a high inhibitory activity on collagenase. This suggests a possible utilization of this substance as a topical agent to reduce the skin ageing process.

1. Introduction

Centella asiatica (L.) Urban, previously also named *Hydrocotyle asiatica* L., and commonly referred to as Indian pennywort and gotu kola, is an herbaceous plant belonging to the A*piaceae* family with great medicinal value. It has been used in Traditional Medicine as an antipyretic, diuretic and antibacterial drug and in the treatment of skin disease, vein insufficiency and mental disorders [1–3]. It is native to Asia and mainly found in India, Pakistan and Madagascar, but the plant also grows in tropical and equatorial Africa, America and the tropical regions of Oceania. It has been widely cultivated as a vegetable or spice in China, Southeast Asia, India, Sri Lanka, Africa and Oceanic countries.

Recently, cosmetic preparations obtained from this plant are worldwide on the market for the treatment of cellulite [4]. The main bioactive compounds of *C. asiatica* include polyacetylenes, triterpenoid saponins (asiaticoside, madecassoside) and their respective sapogenins (asiatic and madecassic acid).

Due to the great demand for these major compounds, many researchers have attempted to overproduce them through the *in vitro* culture technique [5–7].

In our laboratory, *Centella* cell cultures were prepared with the aim of producing asiaticoside and madecassicoside, but besides low quantities of these products, unexpected considerable amounts of caffeoyl derivatives were found. After extraction, four caffeoyl derivatives were isolated and identified.

The main product corresponded to 3,5-*O*-dicaffeoyl-4-*O*-malonilquinic acid (**1**), a new compound for which we propose the name of irbic acid. The minor ones were the known 3,5-*O*-dicaffeoylquinic acid, chlorogenic acid and triferulic acid (**2**). The structures of all these compounds have been elucidated by means of HR-MS and NMR and by comparison with known samples.

Surprisingly, the presence of phenolic compounds, strongly absorbing in the UV range between 300 and 330 nm has never

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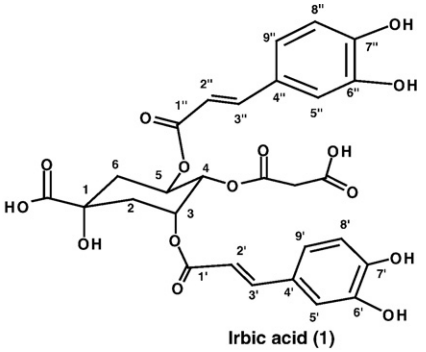
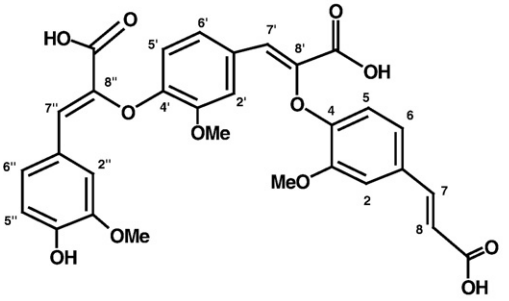
been observed in *C. asiatica* plant extracts, even if the plant is present in the herbal market since at least two decades.



Irbic acid (1)



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3. Results and discussion

3.1. Structural studies

Before extraction, ascorbic acid was added to the cell cultures of *C. asiatica* in order to prevent oxidation by air of the caffeoyl derivatives causing browning of the solution.

Irbic acid (**1**) was isolated from cell cultures by extraction with EtOH and purification by column chromatographies on LH20 and RP-C18 resins.

Compound **1**, obtained as a white amorphous powder, showed a UV spectrum having its maximum absorption at 328 nm and a typical shape pointing out a caffeoyl derivative. A second peak at 220 nm, absent in the phenylethanoid structures (e.g. verbascoside) and characteristic of the quinic acid derivatives, was present.

The HRESIMS spectra in the positive and negative modes obtained with a FT-ICR instrument (see Experimental) showed a very complex cluster of molecular ions due to extensive cationization, accompanied by peaks due to the loss of CO_2 and of a C-3 fragment.

However, from the observation of the peaks at *m/z* 601 in the negative mode and of cationated peaks at *m/z* 663 and 647 in the positive mode, it was possible to attribute to compound **1** the MW of 602 corresponding to the molecular formula C_{28} H $_{26}$ O_{15}.

The homo-, heteronuclear and J-correlated NMR spectra in CD_3OD suggested the presence of a 1,3,4,5-tetrahydroxycycloesan-1-carboxylic acid (quinic acid) substituted at the secondary OHs with two 3-(3′,4′-dihydroxyphenyl)-propenoic acid (caffeic acid) units and with another acyl group. The ^{1}H-NMR spectrum exhibited signals for two caffeoyl moieties (see Experimental), and signals for the quinic acid protons at 5.63 ppm (H-3, ddd , J = 3.3 Hz), 5.23 ppm (H-4, dd, J = 10.2 and 3.4 Hz), 5.70 ppm (H-5, ddd, J = 11.5, 10.2 and 4.5 Hz), 2.40 ppm (H-2eq, dd, J = 15.3 and 3.46 Hz), 2.09 ppm (H-2ax, dd, J = 15.5 and 3.1 Hz) and 2.21 ppm (H-5eq and H-5ax, apparent broad d, J = 11 Hz).

Considering the molecular formula, the presence of a quinic acid moiety and of two caffeic residues, a $C_3H_2O_3$ unit was missing, pointing for a malonic acid residue for the remaining acil substituent.

However, in spite of careful inspection of ^{1}H- and ^{13}C-NMR spectra, no evidences for an additional methylene signal could be detected. By chance, changing the NMR solvent from CD_3OD to Py-D_5 we could find in the ^{1}H spectrum a broad singlet at 3.62 ppm and in the ^{13}C spectrum a signal at 42.87 ppm that were clearly attributed to the methylene of the malonic acid residue.

A plausible explanation of the lack of methylene signal in CD_3OD is a rapid exchange of the active methylene protons with the mobile deuterons of the solvent.

The clue that allowed to determine unambiguously the position of the caffeoyl moieties at C-3 and C-5 was the observation that a sample of **1** in CD_3OD suffered a spontaneous hydrolysis of the malonic unit to give an almost equimolar mixture of **1** and 3,5-*O*-dicaffeoylquinic acid. Subsequently, a mixture of 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid was formed as a result of an internal migration process.

Thus, after one week, the ^{1}H-NMR spectrum showed in addition to the signals of **1**, new signals at 3.92 ppm (H-4, dd, J = 10.1 and 3.5 Hz), 5.40 ppm (H-3, ddd, J = 3.2 Hz), 5.55 ppm (H-5, td, J = 10.3 and 5.4 Hz), These values are in complete agreement with those of the quinic acid moiety of 3,5-*O*-dicaffeoylquinic acid [8]. After 6 weeks the signals of **1** completely disappeared and together with those of 3,5-*O*-dicaffeoylquinic acid, signals appeared at 4.32 ppm (H-3, ddd, J = 3.0 Hz), 5.12 ppm (H-4, dd, J = 10.3 and 3.0 Hz), 5.66 ppm (H-5, td, J = 10.5 and 6.2 Hz), due to 4,5-*O*-dicaffeoylquinic acid [9]. Finally, complete hydrolysis with MeOH furnished quinic acid methyl ester, identical to an authentic sample.

A plausible structure of a 3,5-*O*-dicaffeoyl-4-*O*-malonyl quinic acid has been attributed to one component present in a total of 53 compounds found in the whole plant extract of *Erigeron breviscapus* on the basis of HPLC analysis, MS^n data and without isolation [10].This paper is therefore the first report on the isolation and structural and chemical characterization of irbic acid.

3,5-*O*-Dicaffeoylquinic acid and chlorogenic acid were identified by comparison of their NMR spectra with those of authentic samples, respectively.

Compound 2 showed UV maxima at 294 and 314 nm characteristic of ferulic acid structures. The HR-ESI-MS spectrum in the negative mode showed a $[M-H]^-$ ion at *m/z* 577.13446, corresponding to a MW of 578 and to a molecular formula of $C_{30}H_{26}O_{12}$ [calc 577.13515]. This was confirmed by the positive spectrum which showed sodium adducts at *m/z* 601.13204, [for $C_{30}H_{26}O_{12}Na^+$, calc 601.13208] , 623.11354 [for $C_{30}H_{25}O_{12}Na_2^+$, calc 623.11359] and 645.09560 [for $C_{30}H_{24}O_{12}Na_3^+$, calc 645.09571].

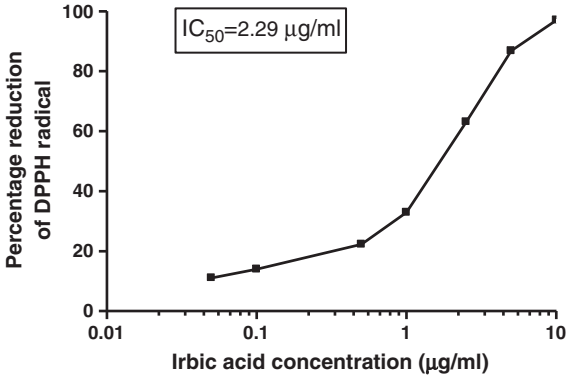

Fig. 1. DPPH radical scavenging capacity (expresses as % DPPH reduction) in the presence of different doses of irbic acid.

The ^{1}H-NMR spectrum (see Experimental) showed an AB system for the vinilic protons of one ferulic acid residue, three ABX systems for the benzene protons of three unsubstituted ferulic acids and two singlets for the β-vinilic protons of α-substituted ferulic acids. In spite of some differences due to the solvent used, these data are in agreement with those reported for a triferulic acid (4-*O*-8′/4′-*O*-8″-didehydrotriferulic acid) previously isolated from saponified maize bran [11]. Therefore this structure is attributed to compound 2.

3.2. Biological activities

An initial screening for the biological activities associated to the isolated irbic acid was the antioxidant and radical scavenging activity evaluated by the DPPH assay. In Fig. 1 the percentage of DPPH reduction is reported as a function of increasing concentrations of this compound in the range from 0.05 µg/ml to 10 µg/ml. From the plotted graph, IC_{50} values were obtained. Irbic acid showed an IC_{50} value of 2.29 µg/ml, which is comparable to that of ascorbic acid and caffeic acid (2.1 µg/ml and 1.15 µg/ml, respectively), and much lower than that of resveratrol (10.96 µg/ml) and rutin (16.47 µg/ml), used as positive controls. Thus, compound 1 turned out to have a very high radical scavenging efficacy. It can result to be even more effective than ascorbic acid, since the antioxidant activity of caffeic acid derivatives is maintained at very low concentrations and virtually no pro-oxidant activity has been found for the quinic acid esterified derivatives of caffeic acid even in the presence of copper ions, as reported by Grace and Logan [12].

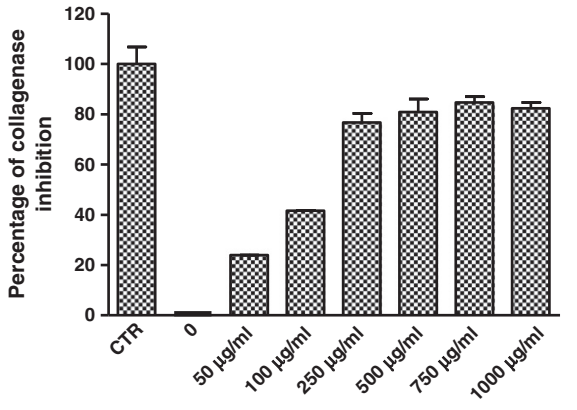

Fig. 2. Percent of collagenase inhibition by different concentrations of irbic acid. CTR = negative control.

Skin anti-aging activity, as measured by the collagenase inhibition assay, was also evaluated in the presence of increasing concentrations of irbic acid. In this assay, a type 1 collagen gel is incubated with the enzyme collagenase and with a specific test compound. If the test substance inhibits the activity of collagenase, the collagen gel will not be degraded, and the amount of this non-degraded form is estimated through the spectrophotometric analysis of the hydroxyproline content, which represents the 11–12% of total amino acids content in collagen. Thus, the amount of hydroxyproline is indirectly proportional to collagenase activity.

As shown in Fig. 2, irbic acid exerts a clear and significant inhibitory effect on collagenase activity. The inhibition is strictly dose-dependent in the range 50–250 µg/ml, thereafter remaining constant.

The degradation of the collagen matrix in the dermal skin layers by external agents such as UV light or as a consequence of physiological ageing is one of the major factors bringing to wrinkle formation. Thus, the ability of irbic acid to significantly inhibit collagenase activity, together with the small size and the hydrophobicity of the molecule, represents an important finding, since it suggests a possible utilization of this substance as a topical agent to reduce the skin ageing process.

Triferulic acid is also a previously described and structurally identified molecule, but has been little tested for its biological properties. In the DPPH antioxidant test, triferulic acid has a much lower antioxidant capacity compared with irbic acid (data not shown) and this result appears to be correlated to the covalent links of the phenolic hydroxy moieties of the caffeic acids.

4. Experimental

4.1. General methods

NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) on a Bruker instrument. Chemical shifts are quoted in δ (ppm) and coupling constants are in Hz.

HR-ESI-MS spectra were acquired with a FT-ICR Bruker Daltonics Apex II instrument equipped with a 4.7 Tesla cryomagnet. Compounds were dissolved in MeOH/H_2O.

HPLC analyses were carried out using an Agilent Technologies mod HP1100 instrument equipped with a diode array detector. Separations were performed on a Luna $C_{18}(2)$ column (3 µm particles, 4.6×250 mm; Phenomenex) in the following conditions: solvent A: H_3PO_4 0.1% added with 0.5% solvent B. Solvent B: H_3PO_4 0.1% in CH_3CN added with 0.5% solvent A. Gradient from 20 to 40% solvent A in B. Flow 0.8 mL/min. Column chromatographies were performed on medium pressure Büchi preparative columns filled with lypophilic Amberlite XAD-4 (Rohm and Haas), Sephadex LH-20 (Pharmacia) or RP-C18 (Merck).

4.2. Cell cultures

Cell cultures of C.*asiatica* were obtained from plants grown in a glass house of the Botanical Garden of the University of Bologna in June 2008. A voucher specimen (no. BOLO 0500914) is kept in the Herbarium of the Department of Biology of University of Bologna (BOLO).

To obtain calli, leaves were surface-sterilized with a 15% commercial bleach solution (final concentration of active chlorine 5%), dissected in small fragments and placed on Petri dish containing solidified Gamborg B5 (13) medium supplemented with with 20 g/L sucrose, 1 g/L plant peptone, 1 mg/L kinetin, 1 mg/L naphthalenacetic acid and 0.2 mg/L indolacetic acid at pH 6.5. Explants were incubated at 25 °C in the dark and, after the callus induction, were subcultured every 30 days.

Calli grown on solid Gamborg B5 medium, as described above, were subjected to subculture for at least 3 months and subsequently were used to inoculate Erlenmeyer flasks of 1 L volume, with the final liquid Gamborg B5 medium content of 250 mL for each flask. After 14 days at 25 °C in the dark, the cells were collected and the secondary metabolites extracted.

4.3. Extraction and isolation

15-days old cell cultures (15 L) were filtered and the medium discarded. Cells were added with 15 g of solid ascorbic acid, extracted with two volumes of EtOH and homogenized. After centrifugation, the extract was concentrated under reduced pressure. The phenolic compounds present in the aqueous residue were quantitatively recovered by solid phase extraction using a column (7×50 cm) containing 1.5 kg of XAD-4 resin suspended in 5% aqueous HCOOH. The column was washed with H_2O and eluted with 80% EtOH. Fractions containing caffeic derivatives were concentrated under reduced pressure and the aqueous residue freeze dried, yielding 25.72 g of grey powder. By HPLC analyses, the material showed the presence of 6.50 g of **1**, 2.71 g of 3,5-*O*-dicaffeoylquinic acid, 2.98 g of **2** and 1.12 g of chlorogenic acid, all expressed as chlorogenic acid.

In order to separate the different caffeic derivatives, the powder was resuspended in H_2O and completely dissolved with a few drops of conc. NH_4OH. Solution was submitted to Sephadex LH20 CC (8×24 cm) suspended in aqueous 15% EtOH added with 1% HCOOH. The column was first eluted with the same solvent, and fractions (100 mL/each) were collected and analyzed by HPLC. Fractions 45–53 were pooled and lyophilized obtaining mg 753 of pure chlorogenic acid. Fractions 63–71 gave 2.37 g of 3,5-*O*-dicaffeoylquinic acid. Elution was continued with aqueous EtOH 30% in HCOOH 1% and fractions 79–98 gave 9.53 g of material containing a mixture of compounds **1** and **2**. In order to separate these products, the powder obtained by freeze drying fractions 79–98 was submitted to a second column chromatography in reverse phase on RP-C18 Lichrosphere (Merck). A column (5×40 cm), filled with resin suspended in aqueous CH_3CN 1% added with HCOOH 1%, was used. The column was eluted with increasing amounts of CH_3CN added of 1% HCOOH and 150 mL fractions were collected. Pure compound **1** (4.25 g) and pure compound **2** (1.95 g) were recovered in the fractions with 20% CH_3CN and 50% respectively.

4.3.1. 3,5-O-dicaffeoyl-4-O-malonylquinic acid (**1**)

White-grey amorphous powder; $[\alpha]_D^{28}$−220 (c 0.18, MeOH); UV (MeOH) λ_{max} 245sh, 299, 327 nm; + NaOH 265sh, 309, 373; ^{1}H NMR (CD_3OD) 7.64 and 6.26 (AB system, J=15.1, H-3′ and H-2′ or H-3″ and H-2″), 7.55 and 6.39 (AB system, J=15.9, H-3″ and H-2″ or H-3′ and H-2′), 7.10 and 7.07 (each 1 H, J=1.96, H-5′ and H-5″), 6.98 and 6.90 (each 1 H, dd, J=7.8, 2.0, H-6′ and H-6″), 6.80 and 6.78 (each 1 H, d, J=7.9, H-5′ and H-5″), 5.70 (1 H, ddd, J=11.5, 10.2, 4.5, H-5), 5.63 (1 H, apparent quartet, J=3.3, H-3), 5.23 (1 H, dd, J=10.2, 3.4, H-4), 2.40 (1 H, dd, J=15.3, 3.46, H-2eq), 2.21 (1 H, apparent d, H-5eq and H-5ax), 2.09 (1 H, dd, J=15.5, 3.1, H-2ax); ^{13}C NMR (CD_3OD), selected data: 76.40 (C-1), 75.17 (C-4), 71.53 (C-3), 69.85 (C-5).

^{1}H-NMR (Py-D_5), selected data: 6.11 (2 H, m, H-3 and H-5), 5.79 (1 H, dd, J=7.03, 3.30, H-4), 3.62 (2 H, broad s, malonic methylene); ^{13}C NMR (Py-D_5), selected data: 74.80 (C-1), 72.82 (C-4), 69.53 and 68.83 (C-3 and C-5), 42.87 (malonic methylene), 38.69 (C-6), 36.90 (C-2).

HRESIMS (negative mode): *m/z* 639.08213 [calcd for $C_{28}H_{24}O_{15}K_1$ (−1), 639.07578]; 633.04917 [calcd for $C_{27}H_{23}O_{13}K_2$ (−1), 633.04183]; 623.10752 [calcd for $C_{28}H_{24}O_{15}Na_1$ (−1), 623.10184]; 617.07414 [calcd for $C_{27}H_{23}O_{13}K_1Na_1$ (−1), 617.06789]; **601.12362 [calcd for $C_{28}H_{25}O_{15}$(−1), 601.11989];** 595.08991 [calcd for $C_{27}H_{24}O_{13}K_1$ (−1), 595.08595]; 579.11503 [calcd for C_{27} H24 $O_{13}Na_1$ (−1), 579.11201]; 575.05831 [calcd for $C_{25}H_{21}O_{12}K_1Na_1$ (−1), calc 575.05733]; 557.13023 [for $C_{27}H_{25}O_{13}$ (−1), calc 575.05733]; 553.07632 [for $C_{25}H_{22}O_{12}K_1$ (−1) calc 553.07538]; 537.10263 (calcd for C_{25} H22 $O_{12}Na_1$ (−1), 537.10144), 515.11975 [calcd for $C_{25}H_{23}O_{12}$ (−1) 515.11975].

HRESIMS (positive mode): *m/z* 717.00445 [(for $C_{28}H_{24}O_{15}K_3$ (+1) calc 717.00209], 701.02885 [for $C_{28}H_{24}O_{15}Na_1K_2$ (+1) calc 701.02815], 685.05479 [for $C_{28}H_{24}O_{15}Na_2K_1$(+1) calc 685.05422] ; 669.08240 [for $C_{28}H_{24}O_{15}Na_3$ (+1) calc 669.08028], **663.07343 [for $C_{28}H_{25}O_{15}Na_1K_1$(+1) calc 663.07227]; 647.10067 [for $C_{28}H_{25}O_{15}Na_2$(+1) calc 647.09834];** 615.02881 [for $C_{25}H_{22}O_{12}$ Na1 K_2 (+1) calc 615.02776]; 599.05572 [for $C_{25}H_{22}O_{12}Na_2K_1$(+1) calc 599.05382], 577.0730 [for $C_{25}H_{23}O_{12}Na_1K_1$ (+1) calc 577.07188]; 561.09932 [for $C_{25}H_{23}$ O12 Na_2 (+1) calc 561.09794].

After one week. ^{1}H NMR (CD_3OD): in addition to the signals of **1**, selected signals due to **3,5-*O*-dicaffeoylquinic acid** are present at 5.56 (1 H, td, J=10.3 and 5.4, H-5), 5.39 (1 H, apparent quartet, J=3.2, H-3), 3.94 (1 H, dd, J=10.0 and 3.6, H-4), 2.31 (1 H, dd, J=14.5 and 3.1, H-2eq), 2.18 (2 H, apparent d, H-5eq and H-5ax), 2.08 (1 H, dd, J=14.6 and 3.7, H-2ax); ^{13}C NMR (CD_3OD), selected data: 76.25 (C-1), 72.20 and 72.10 (C-3 and C-5), 69.80 (C-4).

After six weeks. ^{1}H NMR (CD_3OD): in addition to the signals of **3,5-*O*-dicaffeoylquinic acid**, selected signals due to **4,5-*O*-dicaffeoylquinic acid** are present at: 5.66 (H-5, td, J=10.5, 6.2 Hz), 5.12 (H-4, dd, J=10.3, 3.0 Hz), 4.32 (H-3, ddd, J=3.0 Hz); ^{13}C NMR (CD_3OD): selected data: 75.40 (C-4), 69.50 (C-5), 69.10 (C-3).

4.3.2. 3,5-O-dicaffeoylquinic acid

^{1}H NMR (CD_3OD) selected data: 5.54 (1 H, td, J=10.3, 5.3, H-5), 5.40 (1 H, apparent q, J=3.15, H-3), 3.92 (1 H, dd, J=10.0, 3.4, H-4), 2.31 (1 H, dd, J=14.6, 3.0, H-2 eq), 2.18 (2 H, apparent d, H-5eq and H-5ax), 2.09 (1 H, dd, J=14.7,

3.6, H-2ax). ^{13}C NMR (CD_3OD), selected data: 76.30 (C-1), 72.18 and 72.09 (C-3 and C-5), 69.80 (C-4). For an authentic sample of 3,5-*O*-dicaffeoylquinic acid: 5.52 (1 H, td, $J=10.2$, 5.5, H-5), 5.42 (1 H, apparent q, $J=3.5$, H-3), 3.92 (1 H, dd, $J=9.1$, 3.4, H-4).

4.3.3. Chlorogenic acid

^{1}H NMR (CD_3OD): 7.58 and 6.28 (each 1 H, d, $J=15.9$, H-7′ and H-6′), 7.03 (1 H, d, $J=2.0$, H-2′), 6.95 (1 H, dd, $J=8.2$, 2.0, H-6′), 6.75 (1 H, d, $J=8.3$, H-5′), 5.36 (1 H, dt, $J=10.4$, 5.2, H-5), 4.15 (1 H, apparent q, $J=3.1$, H-3), 3.75 (3 H, s, OMe), 3.70 (1 H, dd, $J=7.7$, 3.4, H-4), 2.2–2.0 (4 H, m. H_2-6 and H_2-2). ^{13}C NMR (CD_3OD), selected data: 76.0 (C-4), 73.6 (C-1), 72.6 (C-5), 67.5 (C-3). For an authentic sample of chlorogenic acid: 5.34 (1 H, dt, $J=10.3$, 5.3, H-5), 4.17 (1 H, apparent q, $J=3.1$, H-3), 3.75 (3 H, s, OMe), 3.68 (1 H, dd, $J=7.8$, 3.5, H-4).

*4.3.4. 4-O-8′/4′-O-8″-Didehydrotriferulic acid (Triferulic acid) (**2**)*

Amorphous solid; UV (MeOH) λ_{max} 245sh, 294, 314 nm; ^{1}H NMR (CD_3OD): 7.63 and 6.42 (each 1 H, d, $J=16.1$, H-7 and H-8), 7.41 and 7.38 (each 1 H, s, H-7 and H-7′), 7.61, 7.37 and 7.34 (each 1 H, d, $J=1.9$, H-2, H-2′ and H-2″), 7.12, 7.10 and 7.07 (each 1 H, dd, $J=8.3$, 1.9, H-6, H-6′ and H-6″), 6.81 and 6.75 (2) (each 1 H, d, $J=8.3$, H-5, H-5′ and H,5″), 3.99, 3,79 and 3.60 (each 3 H, OMe); ^{13}C NMR (CD_3OD): 170.52, 166,76, 166.54, 150.68, 150.21, 149.87, 149.32, 148.97, 148.72, 145.97, 140.41, 138.90, 130.85, 128.97, 128.59, 127.94, 126.51, 125.70, 123.18, 117.97, 116.31, 115.17(3), 114.65, 114.12, 113.28, 56.99, 56.54, 56.15 HRESIMS (negative mode): 577.13446 [for $C_{30}H_{25}O_{12}(-1)$ calc 577.13515], 367.67631, 345.87728; HRESIMS (positive mode): *m/z* 601.13204, [for $C_{30}H_{26}O_{12}Na(+1)$, calc 601.13208], 623.11354 [for $C_{30}H_{25}O_{12}Na_2(+1)$, calc 623.11359], 645.09560 [for $C_{30}H_{24}O_{12}Na_3(1)$, calc 645.09571], 365.10574, 203.05255.

4.4. Biological activities

4.4.1. Antioxidant activity

Free radical scavenging activity was measured using the radical chromogen DPPH (2,2-diphenyl-1-pycryl-hydrazyl) according to the method described by Molyneux [13]. Irbic acid was diluted to different final concentrations of 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 μg/mL and was added to a 0.1 mM DPPH solution.

After 15 min, the absorbance values were spectrophotometrically measured at 517 nm (Shimadzu UV-1601, Japan). A negative control was prepared without the sample.

The percentage of radical reduction (quenching, Q) is expressed as:

$$Q = 100(Abs_{\text{negative ctr}}) - Abs_{\text{sample}})/Abs_{\text{negative control}}$$

Results were expressed as IC_{50} (Inhibitory Concentration) values, defined as the concentration of substrate that induces 50% of DPPH reduction (loss of colour).

4.4.2. Collagenase inhibition assay

The test utilized was that described by Madhan et al. [14]. Specifically, 500 μL of a standard Type I collagen solution (1 mg/mL) were plated on tissue culture dish (35×10 mm) kept at room temperature for 10 min, until solidification of the solution occurred.

The following samples were incubated with the collagen gel:

1. Type IA Collagenase 30 μg/mL (70 U/mL) in TES buffer (positive control)
2. Irbic acid at different dilutions + collagenase 30 μg/mL (70 U/mL), always in TES buffer
3. TES buffer (without enzyme and sample to be tested) as negative control 1 mL of these solutions was added in each culture dish and incubated for 15 h at 37 °C. After this time, the excess medium was discharged and the non-cleaved collagen washed twice with phosphate buffered saline (PBS) 1×. The remaining collagen was hydrolyzed in sealed hydrolysis tubes with 6 N HCl for 15–18 h. The hydrolyzed products were evaporated to dryness with nitrogen gas flux in an evaporator (PIERCE Reacti-Vap III, 18785). The acid free residue was redissolved in a defined volume of TES buffer and the percentage of hydroxyproline was determined using the method of Edwards and O'Brien [15] after preparing a standard curve with this amino acid.

The percentage of collagenase inhibition is expressed as:

$$\left(\left[\mu g/\text{ml hydroxyproline}_{\text{sample}} - \mu g/\text{ml hydroxyproline}_{\text{positive control}}\right) \div \left[\mu g/\text{ml hydroxyproline}_{\text{negative control}}\right)\right] \times 100$$

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